IN THE UNITED STATES DISTRICT COURT

FOR THE DISTRICT OF DELAWARE

10x GENOMICS, INC., Plaintiff, v. CELSEE, INC., Defendant.	Case No. 1:19-cv-00862-CFC-SRF DEMAND FOR JURY TRIAL

SECOND AMENDED COMPLAINT FOR PATENT INFRINGEMENT, UNFAIR COMPETITION, AND FALSE ADVERTISING

Plaintiff 10x Genomics, Inc. ("10x"), for its Second Amended Complaint against Defendant Celsee, Inc. ("Celsee"), alleges as follows:

NATURE OF THE ACTION

1. This is an action for infringement of United States Patent Nos. 10,155,981; 10,240,197; 10,273,541; 10,280,459; 10,392,662; and 10,400,280[1] under the Patent Act, 35 U.S.C. §§1 *et seq.*, including 35 U.S.C. §271; unfair competition and false advertising under Section 43(a) of the Lanham Act, 15 U.S.C. §1125(a); and for related claims under the laws of the State of Delaware.

THE PARTIES

2. 10x Genomics, Inc. is a Delaware corporation with its principal place of business in Pleasanton, California.

3. Celsee, Inc. is a Delaware corporation with its principal place of business in Ann Arbor, Michigan.

[1] Exhibits A–F.

JURISDICTION AND VENUE

4. This civil action arises under the patent laws of the United States, 35 U.S.C. §§ 1, *et seq.*, under the Lanham Act, 15 U.S.C. §§ 1051 *et seq*., and under the Delaware Deceptive Trade Practices Act, 6 Del. C. §§ 2531 *et seq*. This Court has jurisdiction over the subject matter of this action pursuant to 28 U.S.C. §§ 1331 and 1338 as to the claims arising under federal law and pursuant to 28 U.S.C. § 1367 as to the claims arising under state law.

5. This Court has personal jurisdiction over Celsee, and venue is proper in this district pursuant to 28 U.S.C. § 1400(b), because Celsee is a Delaware corporation and thus resides in this district.

BACKGROUND

A. 10x Develops and Commercializes its Groundbreaking Single Cell Technology

6. 10x is a life sciences technology company founded in 2012 in Pleasanton, California by Drs. Serge Saxonov and Benjamin Hindson. 10x is a worldwide leader in genomics, the comprehensive study of biological systems at a molecular and cellular level. Since its founding in 2012, 10x has invested hundreds of thousands of work hours and over a hundred million dollars to invent, design, develop, market, and sell its proprietary line of products for understanding biology at unprecedented resolution and scale.

7. 10x launched its award-winning GemCode product in February 2015, its successor Chromium product line in early 2016, and its Next GEM technology in 2019. 10x's Chromium product line includes a suite of several integrated solutions, each comprising the Chromium Controller and Chromium Connect instruments, biochemical reagents, and analysis and visualization software. Current Chromium products include Chromium Controller, Chromium Connect, the Chromium Single Cell Gene Expression solution, the Chromium Single Cell Immune Profiling solution, the Chromium Single Cell ATAC solution, the Chromium

Single Cell CNV solution, and the Chromium Genomic/Exome solutions. Each of these solutions is designed to interrogate a major class of biological information that is impactful for researchers.

8. For example, the Chromium Single Cell Gene Expression solution provides researchers with the ability to measure gene activity on a cell-by-cell basis, for massive numbers of cells in a single experiment. This approach overcomes limitations of prior tools that analyzed biological samples by obtaining measurements that were averages over large numbers of cells. Such averaged or "bulk" approaches lose critical biological information. For example, in the context of cancer biology, a tumor can consist of a heterogeneous population of cells, some healthy and some cancerous cells, the latter of which may actually consist of genetically distinct tumor cell subpopulations that are each susceptible to different therapeutics. This complexity is easily lost using bulk approaches but can be fully captured using 10x's solutions.

9. In the four years since 10x's first product launch in 2015, 10x's products have won wide acclaim and commercial success. 10x has achieved an installed base of over 1,000 Chromium instruments around the world, including in 93 of the top 100 research institutions and 13 of the top 15 biopharmaceutical companies. Annual sales of 10x products exceeded $140 million in 2018. Over 600 peer-reviewed scientific articles have been published based on data generated from 10x products, including over 100 articles in top journals such as *Cell*, *Science*, and *Nature*. 10x is now an established market leader in single cell genomics, an emerging field that *Science* magazine hailed as the "2018 Breakthrough of the Year."

10. 10x has protected some, but not all, of its innovations with patents duly issued by the United States Patent and Trademark Office. 10x also has other confidential and proprietary technical information, as well as financial and business information, including but not limited to

confidential information about 10x's costs, materials, vendors, business plans, and product roadmaps, all of which is critical to its success.

11. 10x takes great care in guarding its proprietary information and technologies. For example, 10x requires all employees to sign robust confidentiality agreements as conditions of their employment at 10x. Access to 10x's offices is strictly controlled and monitored. 10x's computer systems are password and firewall protected, so that only authorized users can access the company's systems.

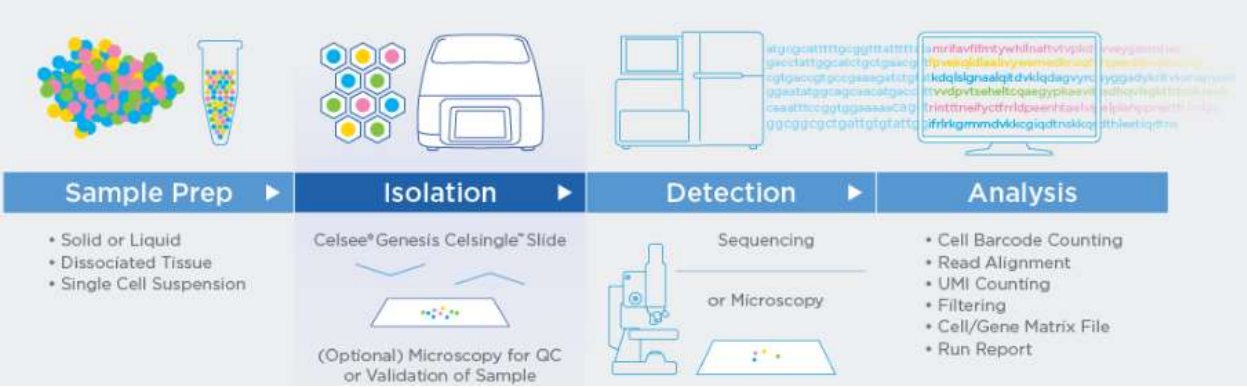
B. The Infringing Celsee Products

12. Roughly three years after 10x launched the Chromium product line, Celsee introduced the Genesis System. Celsee has positioned the Genesis System to compete with 10x's Chromium product line.

13. The Genesis System attempts to enable users to analyze molecular signatures at the single cell level.

14. The Genesis System is designed to capture and isolate single cells. Once isolated, the cells are paired with a unique cellular barcode and unique molecular indices for applications such as gene expression or protein quantitation. *See* https://www.celsee.com/systems/

15. The dual tagging allows the user to track a molecule of interest and the cell of origin of that molecule. The user can therefore detect and quantify the level of the molecule of interest in single cells by sequencing. *See* https://www.celsee.com/systems/



16. Celsee states that such an approach "has the potential to provide a 10x increase in cell throughput compared with other technologies." *See* https://www.celsee.com/systems/

17. Celsee employs what it refers to as Celsingle Technology using Celsingle Slides, which allow a single cell to be paired with a unique cellular barcode and unique molecular indices for desired applications.

18. Celsee has been using, making, marketing, and selling its Genesis System and associated accessories and reagents (collectively the "Genesis Platform"), including Celsingle Technology and Celsingle Slides, since at least mid-2018.

19. Celsee was built to copy 10x's technology and on information and belief has been monitoring its progress, including its patent filings. On information and belief, Celsee instructs and encourages its employees to market Celsee products as superior to the single cell analysis

platform offered by 10x. Celsee does not provide its employees with any substantive support, empirical data, basis, or reasoning for such an assertion.

C. Celsee Aggressively Recruits 10x Employees

20. Celsee's entire business model is aimed at capturing market share and diverting business from 10x by copying 10x's technology and business plans, making false and misleading comparisons between Celsee's product and 10x's, and undercutting 10x on price.

21. Celsee has been recruiting 10x personnel to join Celsee. Based on preliminary forensic analysis, and on information and belief, several of these former 10x employees copied confidential 10x information shortly before they left 10x and went to Celsee.

22. Simply by way of example, one senior marketing manager at 10x helped to prepare a proprietary, non-public "product roadmap" setting out extensive confidential information about 10x's business plan and competitive strategy. The employee left 10x in November 2018 to join Celsee. Thereafter, non-public pricing data from this product roadmap appeared in price-comparison slides in Celsee marketing materials.

23. Another senior marketing manager at 10x left for Celsee in February 2019. Upon departure from 10x, the employee was expressly and repeatedly instructed to return a company-issued computer to 10x with the files on the hard drive intact. Nonetheless, the employee attempted to "wipe" the company-issued computer's hard drive before returning it. Notwithstanding the effort to erase information and files from the company-issued computer, a preliminary forensic analysis revealed confidential and proprietary 10x documents that were sent from the company-issued computer to the employee's personal email.

24. Consistent with its overall approach of copying 10x wholesale, Celsee has even resorted to copying 10x job postings ***verbatim***. For example, in June 2018, 10x announced an available "Protein Engineer" position. By December 2018, Celsee had posted the same "Protein

Engineer" position, copying the 10x announcement—of the ***329*** words that comprise the 10x announcement, ***324*** appear in the Celsee announcement. *See* Exhibits G–H. Similarly, in July 2018, 10x announced an available "Scientist – Cell Biology/Molecular Pathology" position. By December 2018, Celsee had posted the same "Scientist – Cell Biology/Molecular Pathology" position 10x had announced. Of the ***369*** words describing the 10x position, Celsee copied ***361*** of them. *See* Exhibits I–J.

D. Celsee Uses False and Misleading Representations to Market Its Copycat Product

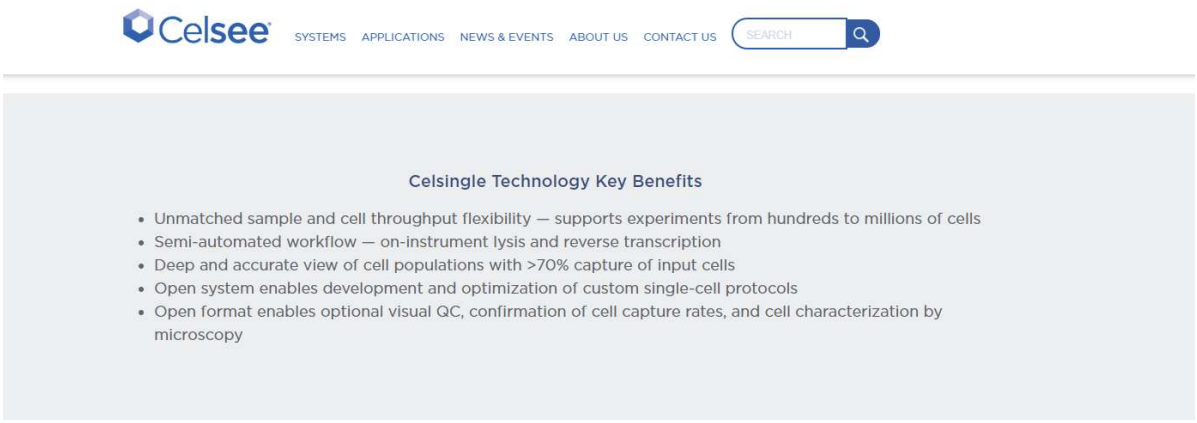
25. In addition to Celsee's attempts to capture market share and divert business from 10x by undercutting 10x on price, Celsee has aggressively marketed the performance characteristics of Celsee's competing product, the Genesis Platform, in false and misleading ways. In particular, in its marketing and promotion materials and on its website, Celsee has made and continues to make false and misleading representations about the performance characteristics of the Genesis Platform.

26. A key criterion on which market participants evaluate the performance of single cell systems is the cell capture rate: a measure of the percentage of input cells that are assayed in each experimental run. The cell capture rate is a critical performance metric relied on by market consumers in evaluating competing single cell analysis products.

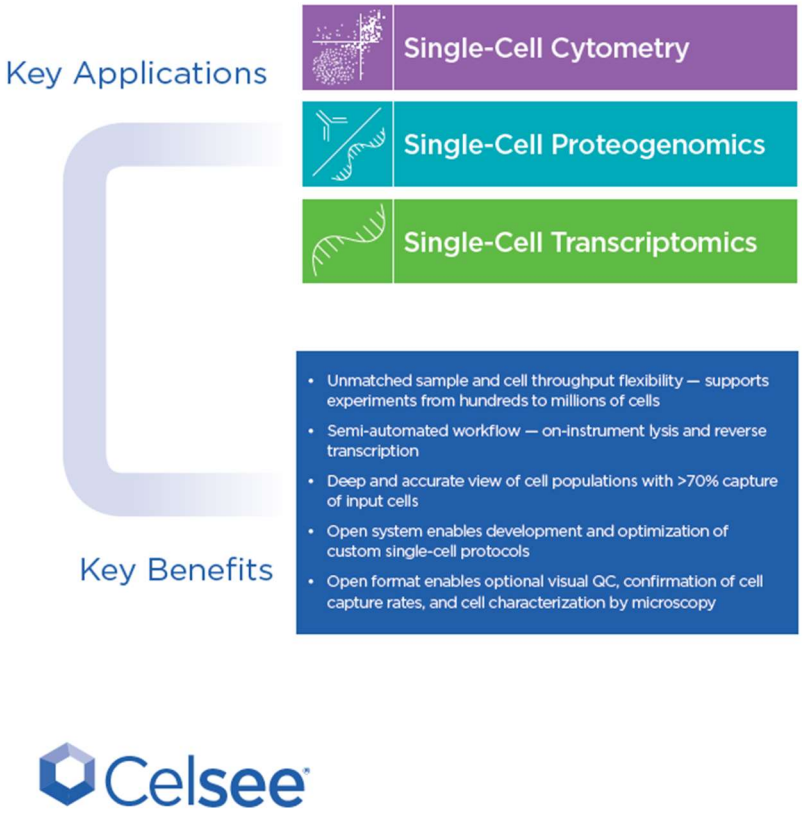
27. 10x's Chromium platform has a 65% cell capture rate. This is considered one of the best cell capture rates in the industry. It is an important basis on which 10x markets the Chromium platform and differentiates itself from the competition.

28. Celsee touts its supposedly superior cell capture rate as one of the key benefits of its system. Indeed, it advertises its system using the tagline: "Because every cell matters." On

its website and in its product literature, Celsee advertises its Genesis Platform as having a cell capture rate of greater than 70% of input cells. *See, e.g.*, Exhibit M:



29. Celsee makes similar claims in product-promotional brochures it directs to target consumers. *See, e.g.*, Exhibit L.



30. On information and belief, these representations are false and misleading, inflating the cell capture efficiency of Celsee's Genesis Platform. Specifically, on information and belief, Celsee measures its cell capture rate as the fraction of cells *paired with beads in the wells of its microwell arrays* to the number of cells *occupying the wells of its microwell arrays*, rather than (as advertised) the number of *input cells* into the system whose sequencing information is *captured* and therefore can be assayed in the experimental run. This method of measuring the cell capture rate is false and misleading in two ways.

31. First, Celsee's purported cell capture rate ignores the substantial numbers of cells that are input into its system, but which reside in the "dead volume" of the system and never make their way into the microwells. Because such cells are never analyzed, they are wasted. In other words, the denominator of the calculation is the number of cells isolated in microwells rather than the number of cells input into the system, and, on information and belief, not all cells input into the system are isolated in microwells. On information and belief, Celsee's purported cell capture rate of greater than 70% of input cells is inaccurate and misleading because these wasted cells are never accounted for.

32. Second, Celsee's purported cell capture rate ignores the substantial numbers of cells paired with beads in its microwell array but whose sequencing information is not recovered from the microwell array. That is, after cells are paired with beads in the Genesis Platform's microwell array, the cells are lysed and nucleic acid molecules from the cells attach to the paired beads. However, a substantial percentage of beads are not recovered from the microwell array. Because cells whose nucleic acid molecules attach to beads that are not recovered from the microwell array are not analyzed through sequencing, they are wasted. In other words, the numerator of the calculation is the number of cells paired with beads rather than the number of

cells whose information is captured. On information and belief, Celsee's purported cell capture rate of greater than 70% of input cells is inaccurate and misleading because these wasted cells are never accounted for.

33. On information and belief, while Celsee advertises its system as having a cell capture rate that is better than that of 10x's products, the reverse is in fact true. On information and belief, the true cell capture rate of Celsee's Genesis Platform is materially less than the rate Celsee promotes it as having, and materially less than the cell capture rate of 10x's Chromium platform.

34. Celsee's misrepresentations regarding the cell capture rate of its Genesis Platform mislead consumers about the performance and efficiency of Celsee's Genesis Platform, and how it compares to 10x's Chromium platform.

35. Reasonable consumers should be able to trust that the representations on Celsee's website and in Celsee's product-promotional materials are true.

36. Celsee's misleading and deceptive practices proximately have caused harm to 10x in that potential sales of 10x's Chromium product line have been and continue to be diverted to Celsee.

THE PATENTS-IN-SUIT

37. Through the development and subsequent making, using, selling, offering for sale, and/or importing of its single cell analysis Genesis Platform, which includes the Genesis System and associated accessories and reagents, Celsee has and continues directly to infringe, contributorily infringe, and/or induce the infringement of:

a. U.S. Patent No. 10,155,981, entitled "Methods for analyzing nucleic acids from single cells," Exhibit A (the "'981 Patent");

b. U.S. Patent No. 10,240,197 entitled "Methods for analyzing nucleic acids

from single cells," Exhibit B (the "'197 Patent");

c. U.S. Patent No. 10,273,541 entitled "Methods and systems for processing polynucleotides," Exhibit C (the "'541 Patent");

d. U.S. Patent No. 10,280,459 entitled "Methods for analyzing nucleic acids from single cells," Exhibit D (the "'459 Patent");

e. U.S. Patent No. 10,392,662 entitled "Methods for analyzing nucleic acids from single cells," Exhibit E (the "'662 Patent"); and

f. U.S. Patent No. 10,400,280 entitled "Methods and systems for processing polynucleotides," Exhibit F (the "'280 Patent").

FIRST CAUSE OF ACTION

(CELSEE'S INFRINGEMENT OF U.S. PATENT NO. 10,155,981)

38. 10x incorporates each of the preceding paragraphs as if fully set forth herein.

39. The '981 Patent issued on December 18, 2018 to Sydney Brenner, Gi Mikawa, Robert Osborne, and Andrew Slatter.

40. By assignment, 10x owns all rights, title, and interest in and to the '981 Patent.

41. Celsee has been on notice of the '981 Patent and its claims since at least the filing of the original Complaint in this action on May 17, 2019, if not earlier.

42. As detailed more fully in Attachment A, Celsee has directly infringed and continues to directly infringe at least claim 1 of the '981 Patent under 35 U.S.C. § 271(a), by using, testing, and demonstrating the Genesis Platform, which includes the Genesis System and its associated accessories and reagents. Celsee has committed and continues to commit these acts of infringement without license or authorization.

43. Celsee, without authority and with knowledge of the '981 patent, has actively induced and continues to actively induce infringement of one or more claims of the '981 patent

under 35 U.S.C. § 271(b) by making and selling the Genesis Platform in the United States and intentionally instructing or otherwise encouraging others, such as scientists working at laboratories that purchase the Genesis Platform, to use the Genesis Platform in the United States in a manner that infringes one or more claims of the '981 patent, as set forth in the claim chart attached as Attachment A. On information and belief, Celsee provided this instruction and encouragement to its actual and prospective customers and end users with the knowledge and intent that doing so would result in the infringement of one or more method claims of the '981 patent by those customers and end users and/or in their performing each step of one or more methods recited in those claims. One or more of Celsee's customers and end users of the Genesis Platform have directly infringed and continue to directly infringe the '981 patent by using the Genesis Platform in accordance with Celsee's instructions and encouragement as set forth in the claim chart attached as Attachment A and incorporated herein by reference.

44. Celsee, without authority, has actively contributed and continues to actively contribute to the infringement of one or more claims of the '981 patent under 35 U.S.C. § 271(c) by selling, offering to sell, importing, or otherwise distributing the Genesis Platform within the United States for use by its customers and end users in a manner that infringes one or more claims of the '981 patent, as set forth in the claim chart attached as Attachment A and incorporated herein by reference.

45. On information and belief, Celsee is and has been aware that the Genesis Platform's nucleic acid sequencer, when used together with the other components of the Genesis Platform, is specially made for use in an infringement of the '981 patent, and has no substantial non-infringing uses. The '981 Patent claims modes of single-cell analysis that involve tagging each molecule being analyzed with separate identifiers pertaining to the molecule itself and the

cell from which it was derived. As detailed in Attachment A, using the Genesis Platform's nucleic acid sequencer requires: (1) isolating single cells in individual microwells, (2) tagging each single cell with unique molecular and cellular identifiers, and then (3) sequencing. As detailed in Attachment A, use of the nucleic acid sequencer as part of the Genesis Platform necessarily infringes the '981 Patent. As further described in Attachment A, the Genesis Platform's nucleic acid sequencer is a separate and distinct feature that has no substantial non-infringing uses and is not a staple article of commerce. The Genesis Platform's nucleic acid sequencer constitutes a material part of the invention.

46. The direct and indirect infringement by Celsee has directly and proximally caused damage to 10x. This infringement entitles 10x to monetary relief in an amount which, by law, cannot be less than a reasonable royalty, together with interest and costs fixed by this Court pursuant to 35 U.S.C. § 284.

47. On information and belief, Celsee regularly monitors 10x's patent portfolio and is aware of the asserted patents.

48. The infringement of the '981 Patent by Celsee is willful and deliberate. At least as of the filing of the Complaint on May 17, 2019, if not earlier, Celsee knew or should have known that its making, using, selling, offering to sell, and/or importing the Genesis Platform, does and will constitute an unjustifiably high risk of infringement of the '981 Patent. Such conduct constitutes, at minimum, willful infringement of the '981 Patent, justifying an award of treble damages pursuant to 35 U.S.C. § 284.

49. Unless Celsee is enjoined from infringing the '981 Patent, 10x will suffer irreparable injury for which damages are an inadequate remedy.

SECOND CAUSE OF ACTION

(CELSEE'S INFRINGEMENT OF U.S. PATENT NO. 10,240,197)

50. 10x incorporates each of the preceding paragraphs as if fully set forth herein.

51. The '197 Patent issued on March 26, 2019 to inventors Sydney Brenner, Gi Mikawa, Robert Osborne, and Andrew Slatter.

52. By assignment, 10x owns all rights, title, and interest in and to the '197 Patent.

53. Celsee has been on notice of the '197 Patent and its claims since at least the filing of the original Complaint in this action on May 17, 2019, if not earlier.

54. As detailed more fully in Attachment B, Celsee has directly infringed and continues to directly infringe at least claim 1 of the '197 Patent under 35 U.S.C. § 271(a), by using, testing, and demonstrating the Genesis Platform, which includes Genesis System and its associated accessories and reagents. Celsee has committed and continues to commit these acts of infringement without license or authorization.

55. Celsee, without authority and with knowledge of the '197 patent, has actively induced and continues to actively induce infringement of one or more claims of the '197 patent under 35 U.S.C. § 271(b) by making and selling the Genesis Platform in the United States and intentionally instructing or otherwise encouraging others, such as scientists working at laboratories that purchase the Genesis Platform, to use the Genesis Platform in the United States in a manner that infringes one or more claims of the '197 patent, as set forth in the claim chart attached as Attachment B. On information and belief, Celsee provided this instruction and encouragement to its actual and prospective customers and end users with the knowledge and intent that doing so would result in the infringement of one or more method claims of the '197 patents by those customers and end users and/or in their performing each step of one or more methods recited in those claims. One or more of Celsee's customers and end users of the

Genesis Platform have directly infringed and continue to directly infringe the '197 patent by using the Genesis Platform in accordance with Celsee's instructions and encouragement as set forth in the claim chart attached as Attachment B and incorporated herein by reference.

56. Celsee, without authority, has actively contributed and continues to actively contribute to the infringement of one or more claims of the '197 patent under 35 U.S.C. § 271(c) by selling, offering to sell, importing, or otherwise distributing the Genesis Platform within the United States for use by its customers and end users in a manner that infringes one or more claims of the '197 patent, as set forth in the claim chart attached as Attachment B and incorporated herein by reference.

57. On information and belief, Celsee is and has been aware that the Genesis Platform's nucleic acid sequencer, when used together with the other components of the Genesis Platform, is specially made for use in an infringement of the '197 patent, and has no substantial non-infringing uses. The '197 Patent claims modes of single-cell analysis that involve tagging each molecule being analyzed with separate identifiers pertaining to the molecule itself and the cell from which it was derived. As detailed in Attachment B, using the Genesis Platform's nucleic acid sequencer requires: (1) isolating single cells in individual microwells, (2) tagging each single cell with unique molecular and cellular identifiers, and then (3) sequencing. As detailed in Attachment B, use of the nucleic acid sequencer as part of the Genesis Platform necessarily infringes the '197 Patent. As further described in Attachment B, the Genesis Platform's nucleic acid sequencer is a separate and distinct feature that has no substantial non-infringing uses and is not a staple article of commerce. The Genesis Platform's nucleic acid sequencer constitutes a material part of the invention.

58. The direct and indirect infringement by Celsee has directly and proximally caused

damage to 10x. This infringement entitles 10x to monetary relief in an amount which, by law, cannot be less than a reasonable royalty, together with interest and costs fixed by this Court pursuant to 35 U.S.C. § 284.

59. On information and belief, Celsee regularly monitors 10x's patent portfolio and is aware of the asserted patents.

60. The infringement of the '197 Patent by Celsee is willful and deliberate. At least as of the filing of the Complaint on May 17, 2019, if not earlier, Celsee knew or should have known that its making, using, selling, offering to sell, and/or importing the Genesis Platform does and will constitute an unjustifiably high risk of infringement of the '197 Patent. Such conduct constitutes, at minimum, willful infringement of the '197 Patent, justifying an award of treble damages pursuant to 35 U.S.C. § 284.

61. Unless Celsee is enjoined from infringing the '197 Patent, 10x will suffer irreparable injury for which damages are an inadequate remedy.

THIRD CAUSE OF ACTION

(CELSEE'S INFRINGEMENT OF U.S. PATENT NO. 10,273,541)

62. 10x incorporates each of the preceding paragraphs as if fully set forth herein.

63. The '541 Patent issued on April 30, 2019 to inventors Benjamin Hindson Christopher Hindson, Michael Schnall-Levin, Kevin Ness, Mirna Jarosz, Serge Saxonov, Paul Hardenbol.

64. By assignment, 10x owns all rights, title, and interest in and to the '541 Patent.

65. Celsee has been on notice of the '541 Patent and its claims since at least the filing of the original Complaint in this action on May 17, 2019, if not earlier.

66. As detailed more fully in Attachment C, Celsee has infringed and continues to infringe at least claim 1 of the '541 Patent under 35 U.S.C. § 271(a), by using, testing, and

demonstrating the Genesis Platform, which includes the Genesis System and its associated accessories and reagents. Celsee has committed and continues to commit these acts of infringement without license or authorization.

67. Celsee, without authority and with knowledge of the '541 patent, has actively induced and continues to actively induce infringement of one or more claims of the '541 patent under 35 U.S.C. § 271(b) by selling the Genesis Platform in the United States and intentionally instructing or otherwise encouraging others, such as scientists working at laboratories that purchase the Genesis Platform, to use the Genesis Platform in the United States in a manner that infringes one or more claims of the '541 patent, as set forth in the claim chart attached as Attachment C. On information and belief, Celsee provided this instruction and encouragement to its actual and prospective customers and end users with the knowledge and intent that doing so would result in the infringement of one or more of the method claims of the '541 patent by those customers and end users and/or in their performance of or more methods recited in those claims. One or more of Celsee's customers and end users of the Genesis Platform have directly infringed and continue to directly infringe the '541 patent by using the Genesis Platform in accordance with Celsee's instructions and encouragement as set forth in the claim chart attached as Attachment C and incorporated herein by reference.

68. Celsee, without authority, has actively contributed and continues to actively contribute to the infringement of one or more claims of the '541 patent under 35 U.S.C. § 271(c) by selling, offering to sell, importing, or otherwise distributing the Genesis Platform within the United States for use by its customers and end users in a manner that infringes one or more claims of the '541 patent, as set forth in the claim chart attached as Attachment C and incorporated herein by reference.

69. On information and belief, Celsee is and has been aware that the Genesis Platform's nucleic acid sequencer, when used together with the other components of the Genesis Platform, is specially made for use in an infringement of the '541 patent, and has no substantial non-infringing uses. The '541 Patent claims modes of single-cell analysis that involve tagging each molecule being analyzed with separate identifiers pertaining to the molecule itself and the cell from which it was derived. As detailed in Attachment C, using the Genesis Platform's nucleic acid sequencer requires: (1) isolating single cells in individual microwells, (2) tagging each single cell with unique molecular and cellular identifiers, and then (3) sequencing. As detailed in Attachment C, use of the nucleic acid sequencer as part of the Genesis Platform necessarily infringes the '541 Patent. As further described in Attachment C, the Genesis Platform's nucleic acid sequencer is a separate and distinct feature that has no substantial non-infringing uses and is not a staple article of commerce. The Genesis Platform's nucleic acid sequencer constitutes a material part of the invention.

70. The direct and indirect infringement by Celsee has directly and proximally caused damage to 10x. This infringement entitles 10x to monetary relief in an amount which, by law, cannot be less than a reasonable royalty, together with interest and costs fixed by this Court pursuant to 35 U.S.C. § 284.

71. On information and belief, Celsee regularly monitors 10x's patent portfolio and is aware of the asserted patents.

72. The infringement of the '541 Patent by Celsee is willful and deliberate. At least as of the filing of the Complaint on May 17, 2019, if not earlier, Celsee knew or should have known that its making, using, selling, offering to sell, and/or importing the Genesis Platform, does and will constitute an unjustifiably high risk of infringement of the '541 Patent. Such

conduct constitutes, at minimum, willful infringement of the '541 Patent, justifying an award of treble damages pursuant to 35 U.S.C. § 284.

73. Unless Celsee is enjoined from infringing the '541 Patent, 10x will suffer irreparable injury for which damages are an inadequate remedy.

FOURTH CAUSE OF ACTION

(CELSEE'S INFRINGEMENT OF U.S. PATENT NO. 10,280,459)

74. 10x incorporates each of the preceding paragraphs as if fully set forth herein.

75. The '459 Patent issued on May 7, 2019 to inventors Sydney Brenner, Gi Mikawa, Robert Osborne, and Andrew Slatter.

76. By assignment, 10x owns all rights, title, and interest in and to the '459 Patent.

77. Celsee has been on notice of the '459 Patent and its claims since at least the filing of the original Complaint in this action on May 17, 2019, if not earlier.

78. As detailed more fully in Attachment D, Celsee has directly infringed and continues to directly infringe at least claim 1 of the '459 Patent under 35 U.S.C. § 271(a), by using, testing, and demonstrating the Genesis Platform, which includes the Genesis System and its associated accessories and reagents. Celsee has committed and continues to commit these acts of infringement without license or authorization.

79. Celsee, without authority and with knowledge of the '459 patent, has actively induced and continues to actively induce infringement of one or more claims of the '459 patent under 35 U.S.C. § 271(b) by selling the Genesis Platform in the United States and intentionally instructing or otherwise encouraging others, such as scientists working at laboratories that purchase the Genesis Platform, to use the Genesis Platform in the United States in a manner that infringes one or more claims of the '459 patent, as set forth in the claim chart attached as Attachment D. On information and belief, Celsee provided this instruction and encouragement

to its actual and prospective customers and end users with the knowledge and intent that doing so would result in the infringement of one or more method claims of the '459 patent by those customers and end users and/or in their performing each step of one or more methods recited in those claims. One or more of Celsee's customers and end users of the Genesis Platform have directly infringed and continue to directly infringe the '459 patent by using the Genesis Platform in accordance with Celsee's instructions and encouragement as set forth in the claim chart attached as Attachment D and incorporated herein by reference.

80. Celsee, without authority, has actively contributed and continues to actively contribute to the infringement of one or more claims of the '459 patent under 35 U.S.C. § 271(c) by selling, offering to sell, importing, or otherwise distributing the Genesis Platform within the United States for use by its customers and end users in a manner that infringes one or more claims of the '459 patent, as set forth in the claim chart attached as Attachment D and incorporated herein by reference.

81. On information and belief, Celsee is and has been aware that the Genesis Platform's nucleic acid sequencer, when used together with the other components of the Genesis Platform, is specially made for use in an infringement of the '459 patent, and has no substantial non-infringing uses. The '459 Patent claims modes of single-cell analysis that involve tagging each molecule being analyzed with separate identifiers pertaining to the molecule itself and the cell from which it was derived. As detailed in Attachment D, using the Genesis Platform's nucleic acid sequencer requires: (1) isolating single cells in individual microwells, (2) tagging each single cell with unique molecular and cellular identifiers, and then (3) sequencing. As detailed in Attachment D, use of the nucleic acid sequencer as part of the Genesis Platform necessarily infringes the '459 Patent. As further described in Attachment D, the Genesis

Platform's nucleic acid sequencer is a separate and distinct feature that has no substantial non-infringing uses and is not a staple article of commerce. The Genesis Platform's nucleic acid sequencer constitutes a material part of the invention.

82. The direct and indirect infringement by Celsee has directly and proximally caused damage to 10x. This infringement entitles 10x to monetary relief in an amount which, by law, cannot be less than a reasonable royalty, together with interest and costs fixed by this Court pursuant to 35 U.S.C. § 284.

83. On information and belief, Celsee regularly monitors 10x's patent portfolio and is aware of the asserted patents.

84. The infringement of the '459 Patent by Celsee is willful and deliberate. At least as of the filing of the Complaint on May 17, 2019, if not earlier, Celsee knew or should have known that its making, using, selling, offering to sell, and/or importing the Genesis Platform, does and will constitute an unjustifiably high risk of infringement of the '459 Patent. Such conduct constitutes, at minimum, willful infringement of the '459 Patent, justifying an award of treble damages pursuant to 35 U.S.C. § 284.

85. Unless Celsee is enjoined from infringing the '459 Patent, 10x will suffer irreparable injury for which damages are an inadequate remedy.

FIFTH CAUSE OF ACTION

(CELSEE'S INFRINGEMENT OF U.S. PATENT NO. 10,392,662)

86. 10x incorporates each of the preceding paragraphs as if fully set forth herein.

87. The '662 Patent issued on August 27, 2019 to inventors Sydney Brenner, Gi Mikawa, Robert Osborne, and Andrew Slatter.

88. By assignment, 10x owns all rights, title, and interest in and to the '662 Patent.

89. Celsee has been on notice of the '662 Patent and its claims since at least October

2, 2019, if not as of the patent's issuance on August 27, 2019.

90. As detailed more fully in Attachment E, Celsee has directly infringed and continues to directly infringe at least claim 1 of the '662 Patent under 35 U.S.C. § 271(a), by making, importing, using, testing, demonstrating, selling, and offering for sale the Genesis Platform, which includes the Genesis System and associated accessories and reagents, including at least the high-density bio beads. Celsee has committed and continues to commit these acts of infringement without license or authorization.

91. The direct infringement by Celsee has directly and proximally caused damage to 10x. This infringement entitles 10x to monetary relief in an amount which, by law, cannot be less than a reasonable royalty, together with interest and costs fixed by this Court pursuant to 35 U.S.C. § 284.

92. The infringement of the '662 Patent by Celsee is willful and deliberate. By August 27, 2019, but at least as of October 2, 2019, Celsee knew or should have known that its making, using, selling, offering to sell, and/or importing the Genesis Platform, including at least the high density Bio Beads, does and will constitute an unjustifiably high risk of infringement of the '662 Patent. Such conduct constitutes, at minimum, willful infringement of the '662 Patent, justifying an award of treble damages pursuant to 35 U.S.C. § 284.

93. Unless Celsee is enjoined from infringing the '662 Patent, 10x will suffer irreparable injury for which damages are an inadequate remedy.

SIXTH CAUSE OF ACTION

(CELSEE'S INFRINGEMENT OF U.S. PATENT NO. 10,400,280)

94. 10x incorporates each of the preceding paragraphs as if fully set forth herein.

95. The '280 Patent issued on September 3, 2019 to inventors Benjamin Hindson Christopher Hindson, Michael Schnall-Levin, Kevin Ness, Mirna Jarosz, Serge Saxonov, and

Paul Hardenbol.

96. By assignment, 10x owns all rights, title, and interest in and to the ’280 Patent.

97. Celsee has been on notice of the ’662 Patent and its claims since at least October 2, 2019, if not as of the patent’s issuance on September 3, 2019.

98. As detailed more fully in Attachment F, Celsee has directly infringed and continues to directly infringe at least claim 1 of the ’280 Patent under 35 U.S.C. § 271(a), by using, testing, and demonstrating the Genesis Platform, which includes the Genesis System and its associated accessories and reagents. Celsee has committed and continues to commit these acts of infringement without license or authorization.

99. Celsee, without authority and with knowledge of the ’280 patent, has actively induced and continues to actively induce infringement of one or more claims of the ’280 patent under 35 U.S.C. § 271(b) by selling the Genesis Platform in the United States and intentionally instructing or otherwise encouraging others, such as scientists working at laboratories that purchase the Genesis Platform, to use the Genesis Platform in the United States in a manner that infringes one or more claims of the ’280 patent, as detailed in the claim charts attached as Attachment F. On information and belief, Celsee provided this instruction and encouragement to its actual and prospective customers and end users with the knowledge and intent that doing so would result in the infringement of one or more method claims of the ’280 patent by those customers and end users and/or in their performing each step of one or more methods recited in those claims. One or more of Celsee’s customers and end users of the Genesis Platform have directly infringed and continue to directly infringe the ’280 patent by using the Genesis Platform in accordance with Celsee’s instructions and encouragement as set forth in the claim chart attached as Attachment F and incorporated herein by reference.

100. Celsee, without authority, has actively contributed and continues to actively contribute to the infringement of one or more claims of the ’280 patent under 35 U.S.C. § 271(c) by selling, offering to sell, importing, or otherwise distributing the Genesis Platform within the United States for use by its customers and end users in a manner that infringes one or more claims of the ’280 patent, as set forth in the claim chart attached as Attachment F and incorporated herein by reference.

101. On information and belief, Celsee is and has been aware that the Genesis Platform’s nucleic acid sequencer, when used together with the other components of the Genesis Platform, is specially made for use in an infringement of the ’280 patent, and has no substantial non-infringing uses. The ’280 Patent claims modes of single-cell analysis that involve tagging each molecule being analyzed with separate identifiers pertaining to the molecule itself and the cell from which it was derived. As detailed in Attachment F, using the Genesis System’s nucleic acid sequencer requires: (1) isolating single cells in individual microwells, (2) tagging each single cell with unique molecular and cellular identifiers, and then (3) sequencing. As detailed in Attachment F, use of the nucleic acid sequencer as part of the Genesis Platform necessarily infringes the ’280 Patent. As further described in Attachment F, the Genesis Platform’s nucleic acid sequencer is a separate and distinct feature that has no substantial non-infringing uses and is not a staple article of commerce. The Genesis Platform’s nucleic acid sequencer constitutes a material part of the invention.

102. The direct and indirect infringement by Celsee has directly and proximally caused damage to 10x. This infringement entitles 10x to monetary relief in an amount which, by law, cannot be less than a reasonable royalty, together with interest and costs fixed by this Court pursuant to 35 U.S.C. § 284.

103. On information and belief, Celsee regularly monitors 10x's patent portfolio and is aware of the asserted patents.

104. The infringement of the '280 Patent by Celsee is willful and deliberate. By September 3, 2019, but at least as of October 2, 2019, Celsee knew or should have known that its making, using, selling, offering to sell, and/or importing the Genesis Platform, does and will constitute an unjustifiably high risk of infringement of the '280 Patent. Such conduct constitutes, at minimum, willful infringement of the '280 Patent, justifying an award of treble damages pursuant to 35 U.S.C. § 284.

105. Unless Celsee is enjoined from infringing the '280 Patent, 10x will suffer irreparable injury for which damages are an inadequate remedy.

SEVENTH CAUSE OF ACTION

(UNFAIR COMPETITION UNDER THE LANHAM ACT)

106. 10x incorporates each of the preceding paragraphs as if fully set forth herein.

107. In its commercial advertising and promotional efforts, Celsee has made false or misleading representations of fact concerning the cell capture rate of its Genesis Platform. Celsee promotes its Genesis Platform as having a cell capture rate of greater than 70% of input cells. This statement is literally false because it reflects the percentage of cells that are input ***and*** that then make it into the microwells of the Genesis System device, and not the percentage of total input cells. This rate therefore does not reflect the true proportion of input cells actually sequenced. Additionally, this statement is literally false because it does not reflect the percentage of cells whose sequencing information is "captured," but only the percentage of cells that are paired with beads in microwells—not all of whose information is captured. Thus, the cell capture rate Celsee promotes its Genesis Platform as having is inaccurate and grossly inflated.

108. Celsee's false or misleading representations of fact concerning the cell capture rate of its Genesis Platform concern the Genesis Platform's nature, characteristics, or qualities.

109. Celsee's false or misleading representations of fact actually deceive or have the tendency to deceive a substantial portion of the target consumers. By promoting an inaccurate and inflated cell capture rate, Celsee has misled target consumers into believing that they are purchasing a genomics system that is more efficient and has better performance than the system they are in fact getting.

110. Celsee's false or misleading representations of fact concerning the cell capture rate of its Genesis Platform are material because they are likely to influence the purchasing decisions of a target consumer, since cell capture rate is an important criterion of system performance and efficiency.

111. Celsee advertises, promotes, sells, and distributes its falsely and misleadingly represented products in interstate commerce.

112. Celsee's Genesis Platform competes directly with 10x's Chromium product line.

113. Celsee's conduct as alleged herein is causing immediate and irreparable harm and injury to 10x, and to its goodwill and reputation, and will continue to both damage 10x through the diversion of sales and confuse the consuming public.

114. As a result of Celsee's wrongful acts, 10x has been damaged in an amount not yet determined or ascertainable. At a minimum, 10x is entitled to injunctive relief, an accounting of Celsee's profits, damages, and costs.

EIGHTH CAUSE OF ACTION

(VIOLATION OF DELAWARE DECEPTIVE TRADE PRACTICES ACT)

115. 10x incorporates each of the preceding paragraphs as if fully set forth herein.

116. In the course of its business, Celsee has made false or misleading representations

of fact concerning the cell capture rate of its Genesis Platform. Celsee promotes its Genesis Platform as having a cell capture rate of greater than 70% of input cells. This statement is literally false because it reflects the percentage of cells that are input ***and*** that then make it into the microwells of the Genesis System device, and not the percentage of total input cells. This rate therefore does not reflect the true proportion of input cells actually sequenced. Additionally, this statement is literally false because it does not reflect the percentage of cells whose sequencing information is "captured," but only the percentage of cells that are paired with beads in microwells—not all of whose information is captured. Thus, the cell capture rate Celsee promotes its Genesis Platform as having is inaccurate and grossly inflated.

117. Celsee's false or misleading representations of fact concerning the cell capture rate of its Genesis Platform create a likelihood of confusion or of misunderstanding, represents that its Genesis Platform is of a particular standard, quality, or grade when it is of another, and represent the Genesis Platform as having characteristics or benefits the Genesis Platform does not have, in violation of subsections 5, 7, and 12 of the Delaware Deceptive Trade Practices Act.

118. Celsee's false or misleading representations of fact actually deceive or have the tendency to deceive a substantial portion of the target consumers. By promoting an inaccurate and inflated cell capture rate, Celsee has misled target consumers into believing that they are purchasing a genomics system that is more efficient and has better performance than the system they are in fact getting.

119. Celsee's false or misleading representations of fact concerning the cell capture rate of its Genesis Platform are material because they are likely to influence the purchasing decisions of a target consumer, since cell capture rate is an important criterion of system performance and efficiency.

120. Celsee's Genesis Platform competes directly with 10x's Chromium product line.

121. Celsee's conduct as alleged herein is causing immediate and irreparable harm and injury to 10x, and to its goodwill and reputation, and will continue to both damage 10x through the diversion of sales and confuse the consuming public.

122. As a result of Celsee's wrongful acts, 10x has been damaged in an amount not yet determined or ascertainable. At a minimum, 10x is entitled to injunctive relief, an accounting of Celsee's profits, damages, and costs.

PRAYER FOR RELIEF

Plaintiff 10x Genomics, Inc. respectfully requests that the Court find in its favor and against Celsee, Inc. and that the Court grant the following relief:

a. For entry of judgment that the '981 Patent, the '197 Patent, the '541 Patent, the '459 Patent, the '662 Patent, and the '280 Patent have been and continue to be directly and/or indirectly infringed by Celsee, either literally or under the doctrine of equivalents;

b. For a declaration that each of the Patents-in-Suit is valid and enforceable;

c. For permanent injunctions enjoining the aforesaid acts of infringement by Celsee, its officers, agents, servants, employees, attorneys, parent and subsidiary entities, assigns and successors in interest, and those persons acting in concert with them, including related individuals and entities, customers, representatives, distributors, and dealers. In the alternative, if the Court finds that an injunction is not warranted, 10x requests an award of post-judgment royalty to compensate for future infringement;

d. For entry of judgment that Celsee has violated 15 U.S.C. § 1125(a) and subsections 5, 7, and 12 the Delaware Deceptive Trade Practices Act by unfairly competing against 10x through false or misleading representations of fact as described herein;

e. For preliminary and permanent injunctive relief prohibiting Celsee, its

officers, agents, servants, employees, attorneys, parent and subsidiary entities, assigns and successors in interest, and those persons acting in concert with them, including related individuals and entities, customers, representatives, distributors, and dealers, from false or misleading representations of fact concerning the cell capture rate of the Genesis System, which relief includes but is not limited to corrective advertising and removal of all false or misleading advertisements;

f. For an accounting of all damages sustained by 10x as the result of the acts of Celsee as alleged herein;

g. For the award to 10x of damages so ascertained, together with pre-judgment interest as provided by law;

h. For the award to 10x of Celsee's profits, gains, and advantages derived from Celsee's unlawful conduct;

i. For entry of judgment that Celsee's infringement is willful, and for an award of treble damages pursuant to 35 U.S.C. § 284;

j. For judgment that this case is exceptional, and for an award of all costs, disbursements, and attorneys' fees pursuant to 35 U.S.C. § 285; and

k. For such other and further legal and/or equitable relief as the Court shall deem just and proper.

/s/ Frederick L. Cottrell, III

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Exhibit A

US010155981B2

(12) **United States Patent**
Brenner et al.

(10) **Patent No.: US 10,155,981 B2**
(45) **Date of Patent: Dec. 18, 2018**

(54) **METHODS FOR ANALYZING NUCLEIC ACIDS FROM SINGLE CELLS**

(71) Applicant: **10X Genomics, Inc.**, Pleasanton, CA (US)

(72) Inventors: **Sydney Brenner**, Ely (GB); **Gi Mikawa**, Great Shelford (GB); **Robert Osborne**, Great Chesterford (GB); **Andrew Slatter**, London (GB)

(73) Assignee: **10X GENOMICS, INC.**, Pleasanton, CA (US)

(*) Notice: Subject to any disclaimer, the term of this patent is extended or adjusted under 35 U.S.C. 154(b) by 0 days.

(21) Appl. No.: **15/677,957**

(22) Filed: **Aug. 15, 2017**

(65) **Prior Publication Data**

US 2018/0080075 A1 Mar. 22, 2018

Related U.S. Application Data

(63) Continuation of application No. 14/792,094, filed on Jul. 6, 2015, which is a continuation of application No. 14/172,694, filed on Feb. 4, 2014, now Pat. No. 9,102,980, which is a continuation of application No. 14/021,790, filed on Sep. 9, 2013, now Pat. No. 8,679,756, which is a continuation of application No. 13/859,450, filed on Apr. 9, 2013, now Pat. No. 8,563,274, which is a continuation of application No. 13/622,872, filed on Sep. 19, 2012, which is a continuation of application No. 13/387,343, filed as application No. PCT/IB2010/002243 on Aug. 13, 2010, now Pat. No. 8,298,767.

(60) Provisional application No. 61/288,792, filed on Dec. 21, 2009, provisional application No. 61/235,595, filed on Aug. 20, 2009.

(51) **Int. Cl.**

C12Q 1/686	(2018.01)
C12N 15/10	(2006.01)
C12Q 1/6874	(2018.01)
C12Q 1/6855	(2018.01)
C12Q 1/6806	(2018.01)

(52) **U.S. Cl.**

CPC ***C12Q 1/6874*** (2013.01); ***C12N 15/1065*** (2013.01); ***C12Q 1/6806*** (2013.01); ***C12Q 1/686*** (2013.01); ***C12Q 1/6855*** (2013.01)

(58) **Field of Classification Search**

CPC C12Q 1/6874; C12N 15/1065
USPC 506/4
See application file for complete search history.

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Primary Examiner — Karla A Dines

(74) *Attorney, Agent, or Firm* — Morgan, Lewis & Bockius LLP

(57) **ABSTRACT**

Aspects of the present invention include analyzing nucleic acids from single cells using methods that include using tagged polynucleotides containing multiplex identifier sequences.

6 Claims, 13 Drawing Sheets

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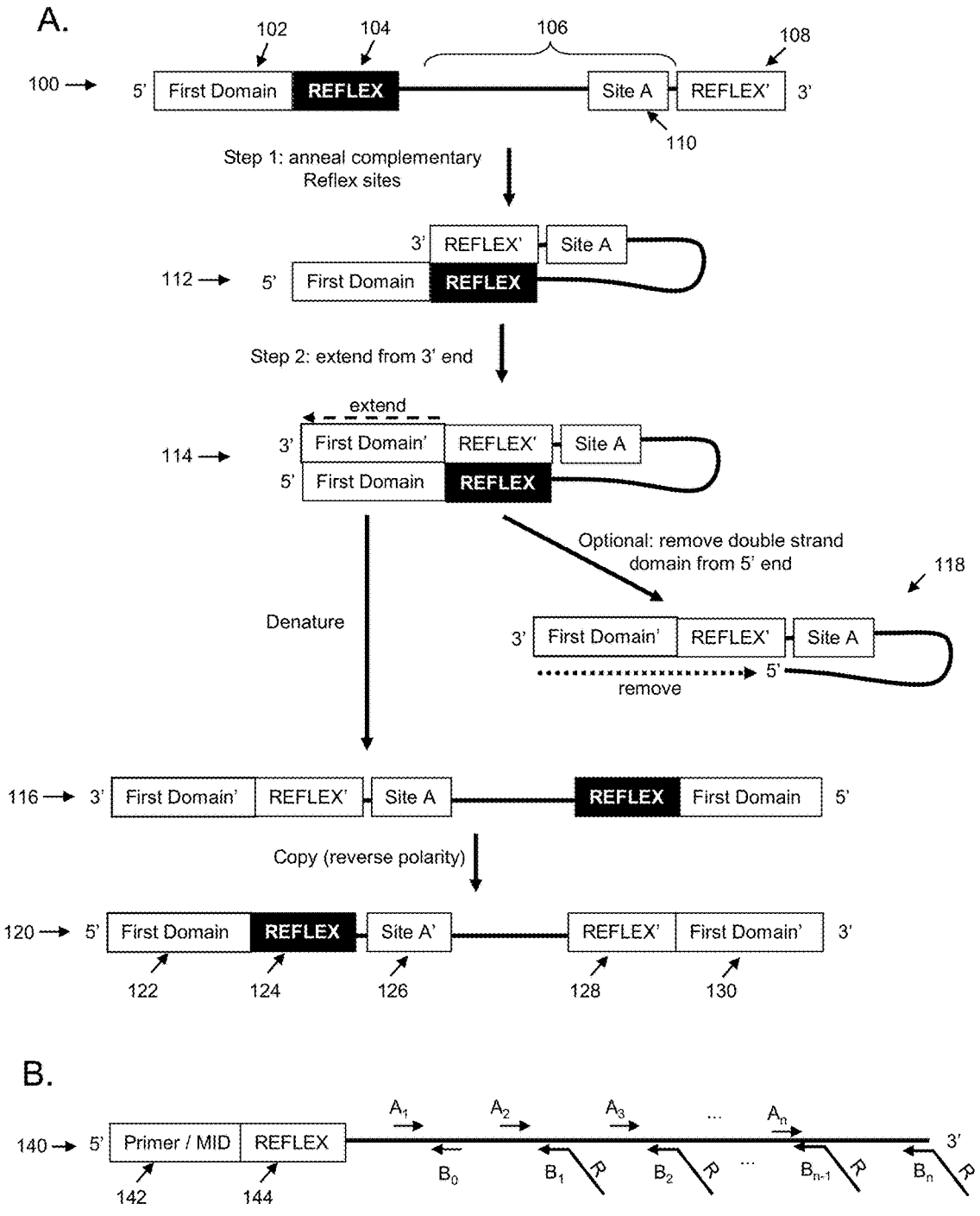

A.
102
104
106
108
100
5'
First Domain
REFLEX
Site A
REFLEX'
3'
110
Step 1: anneal complementary Reflex sites
3'
REFLEX'
Site A
112
5'
First Domain
REFLEX
Step 2: extend from 3' end
extend
114
3'
First Domain'
REFLEX'
Site A
5'
First Domain
REFLEX
Optional: remove double strand domain from 5' end
118
Denature
3'
First Domain'
REFLEX'
Site A
5'
remove
116
3'
First Domain'
REFLEX'
Site A
REFLEX
First Domain
5'
Copy (reverse polarity)
120
5'
First Domain
REFLEX
Site A'
REFLEX'
First Domain'
3'
122
124
126
128
130
B.
140
5'
Primer / MID
REFLEX
142
144
A1
A2
A3
An
B0
B1
B2
Bn-1
Bn
R
3'

Fig. 1

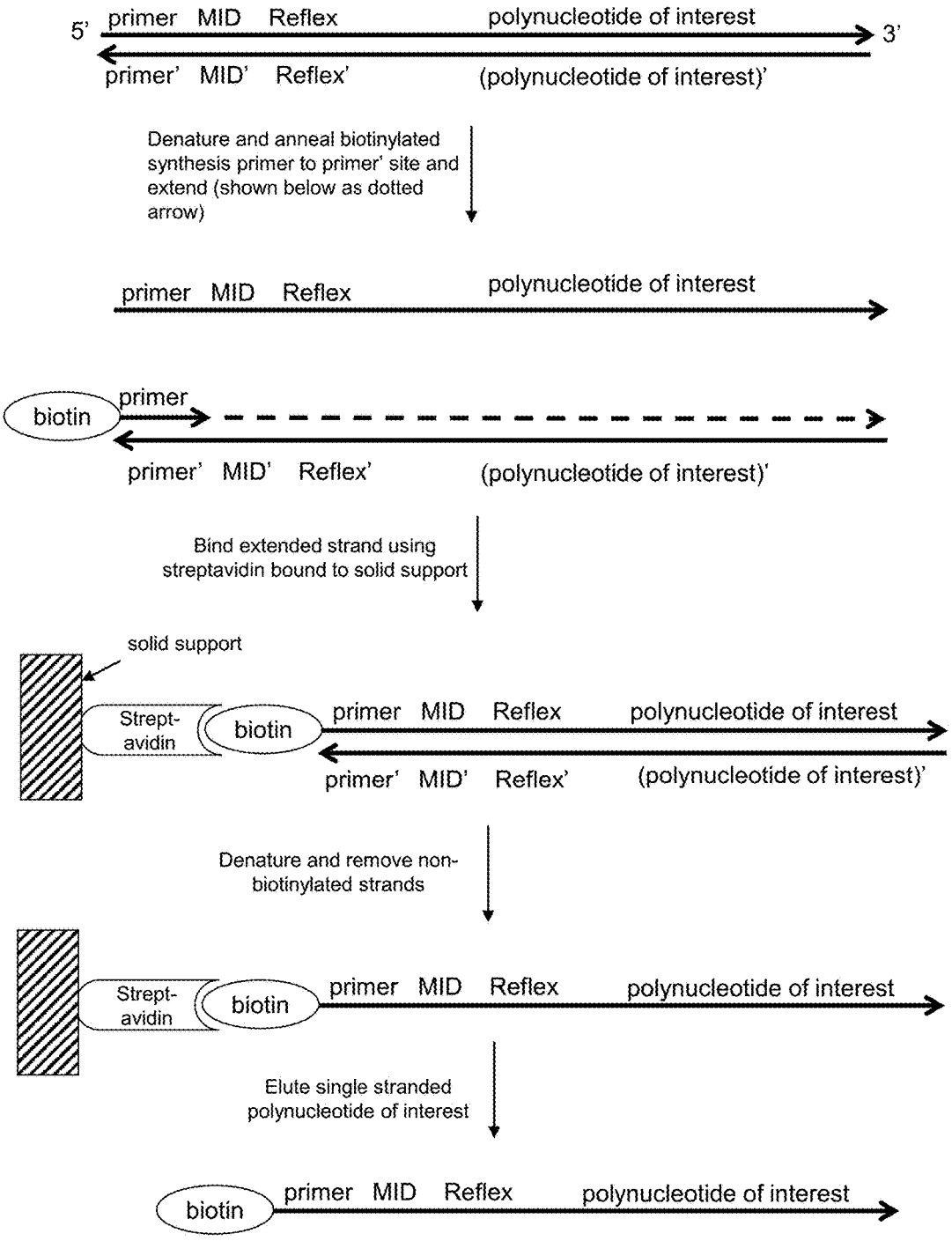
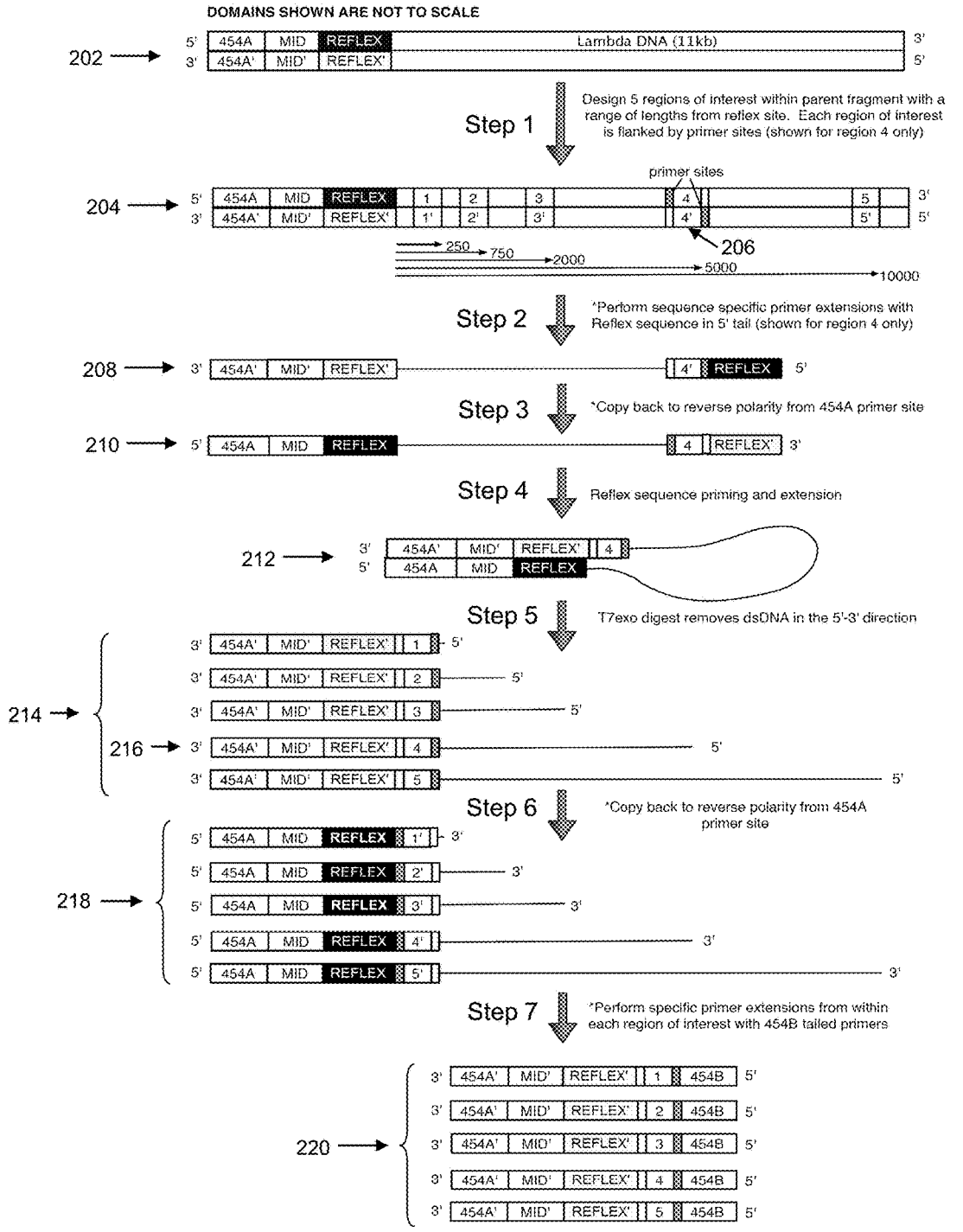

5'
primer MID Reflex
polynucleotide of interest
3'
primer' MID' Reflex'
(polynucleotide of interest)'
Denature and anneal biotinylated synthesis primer to primer' site and extend (shown below as dotted arrow)
primer MID Reflex
polynucleotide of interest
biotin
primer
primer' MID' Reflex'
(polynucleotide of interest)'
Bind extended strand using streptavidin bound to solid support
solid support
Strept-avidin
biotin
primer MID Reflex
polynucleotide of interest
primer' MID' Reflex'
(polynucleotide of interest)'
Denature and remove non-biotinylated strands
Strept-avidin
biotin
primer MID Reflex
polynucleotide of interest
Elute single stranded polynucleotide of interest
biotin
primer MID Reflex
polynucleotide of interest

Fig. 2



Primer extension reactions with * may be performed such that isolation of single strand species is facilitated (e.g., using primers with binding moieties and/or multiple cycles of extension)

Fig. 3

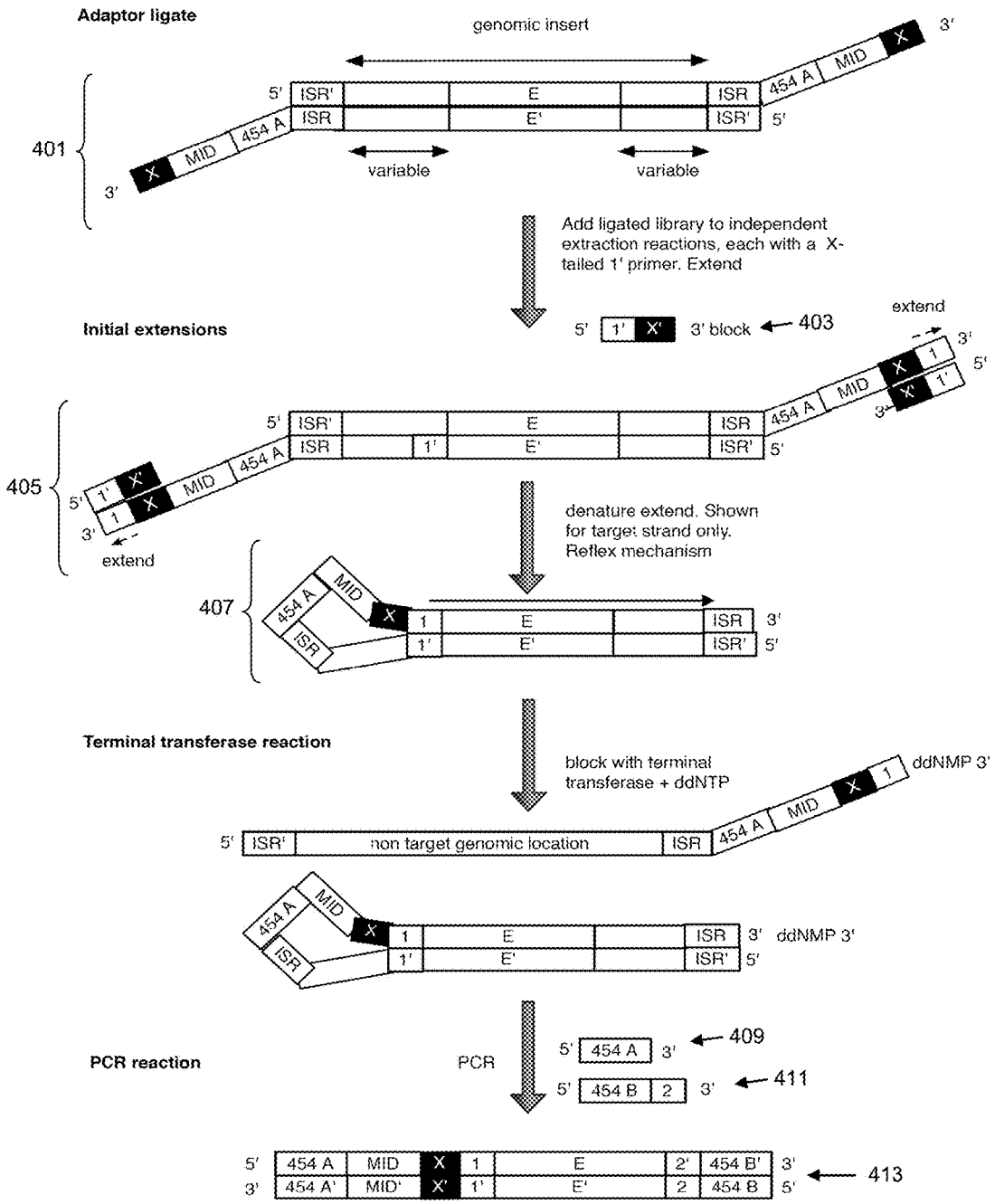

Adaptor ligate
genomic insert
401
5'
3'
ISR'
ISR
E
E'
454 A
MID
X
variable
Add ligated library to independent extraction reactions, each with a X-tailed 1' primer. Extend
Initial extensions
403
3' block
extend
405
1'
1
extend
denature extend. Shown for target strand only. Reflex mechanism
407
Terminal transferase reaction
block with terminal transferase + ddNTP
ddNMP 3'
non target genomic location
PCR reaction
PCR
409
411
454 B
2
413

Fig. 4

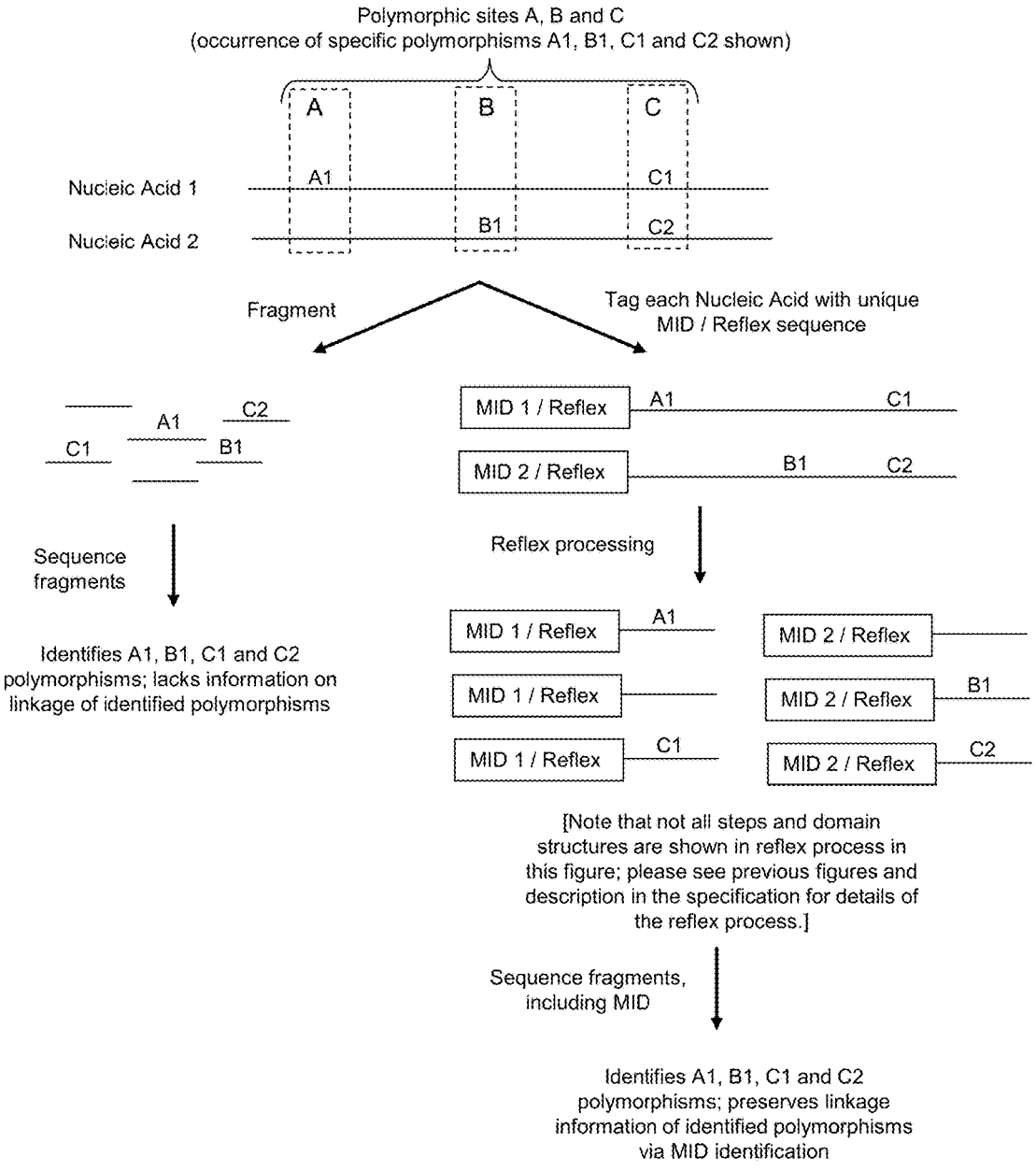
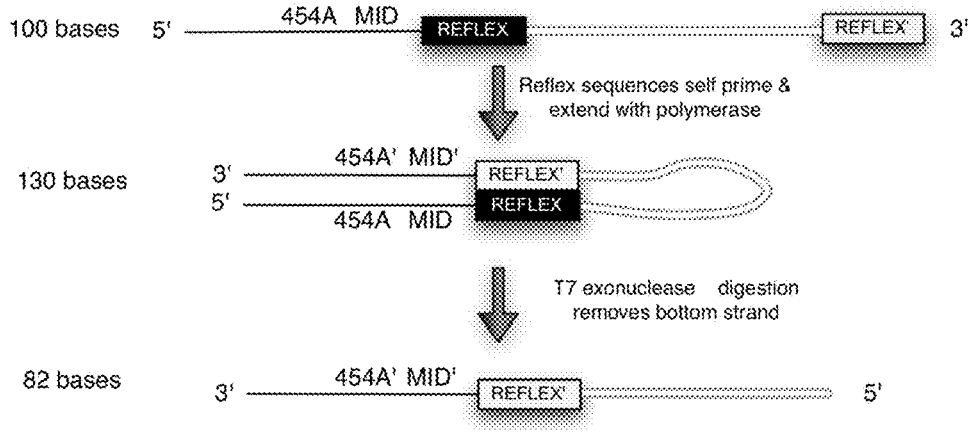
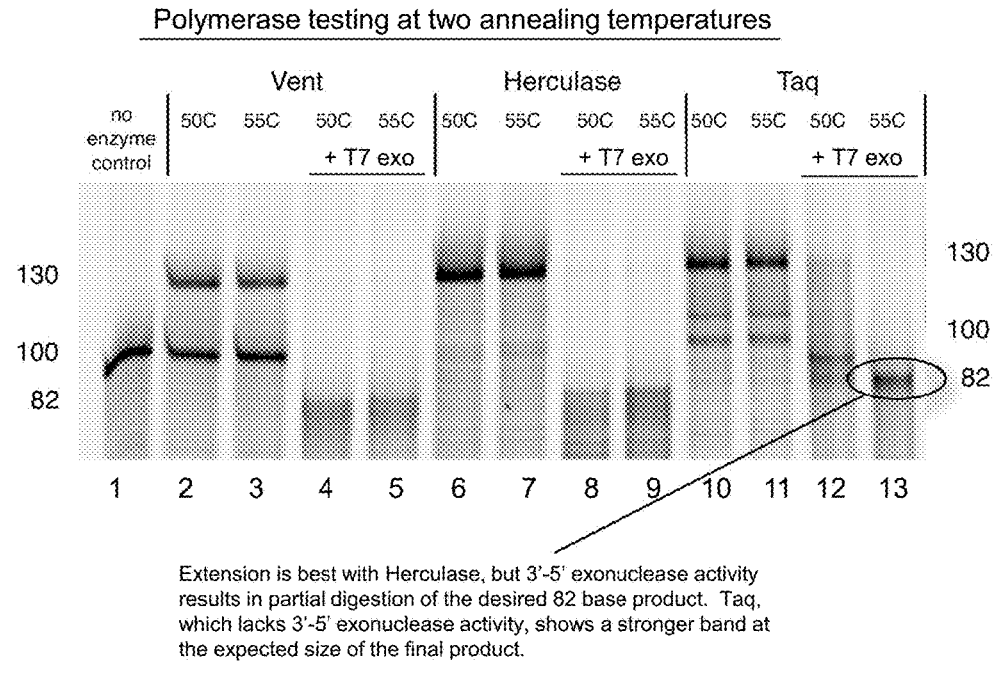

Polymorphic sites A, B and C
(occurrence of specific polymorphisms A1, B1, C1 and C2 shown)
A
B
C
Nucleic Acid 1
A1
C1
Nucleic Acid 2
B1
C2
Fragment
Tag each Nucleic Acid with unique MID / Reflex sequence
A1
C2
C1
B1
Sequence fragments
Identifies A1, B1, C1 and C2 polymorphisms; lacks information on linkage of identified polymorphisms
MID 1 / Reflex
A1
C1
MID 2 / Reflex
B1
C2
Reflex processing
MID 1 / Reflex
A1
MID 2 / Reflex
MID 1 / Reflex
MID 2 / Reflex
B1
MID 1 / Reflex
C1
MID 2 / Reflex
C2
[Note that not all steps and domain structures are shown in reflex process in this figure; please see previous figures and description in the specification for details of the reflex process.]
Sequence fragments, including MID
Identifies A1, B1, C1 and C2 polymorphisms; preserves linkage information of identified polymorphisms via MID identification

Fig. 5

A.



B.



Extension is best with Herculase, but 3'-5' exonuclease activity results in partial digestion of the desired 82 base product. Taq, which lacks 3'-5' exonuclease activity, shows a stronger band at the expected size of the final product.

Fig. 6

A.


5'
454A MID
REFLEX
REFLEX'
3'
+ Competitor
5'
REFLEX'
3'

B.

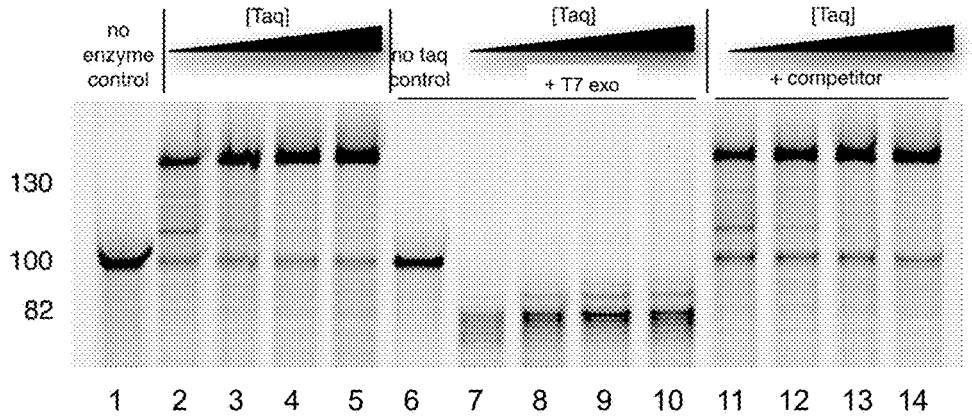
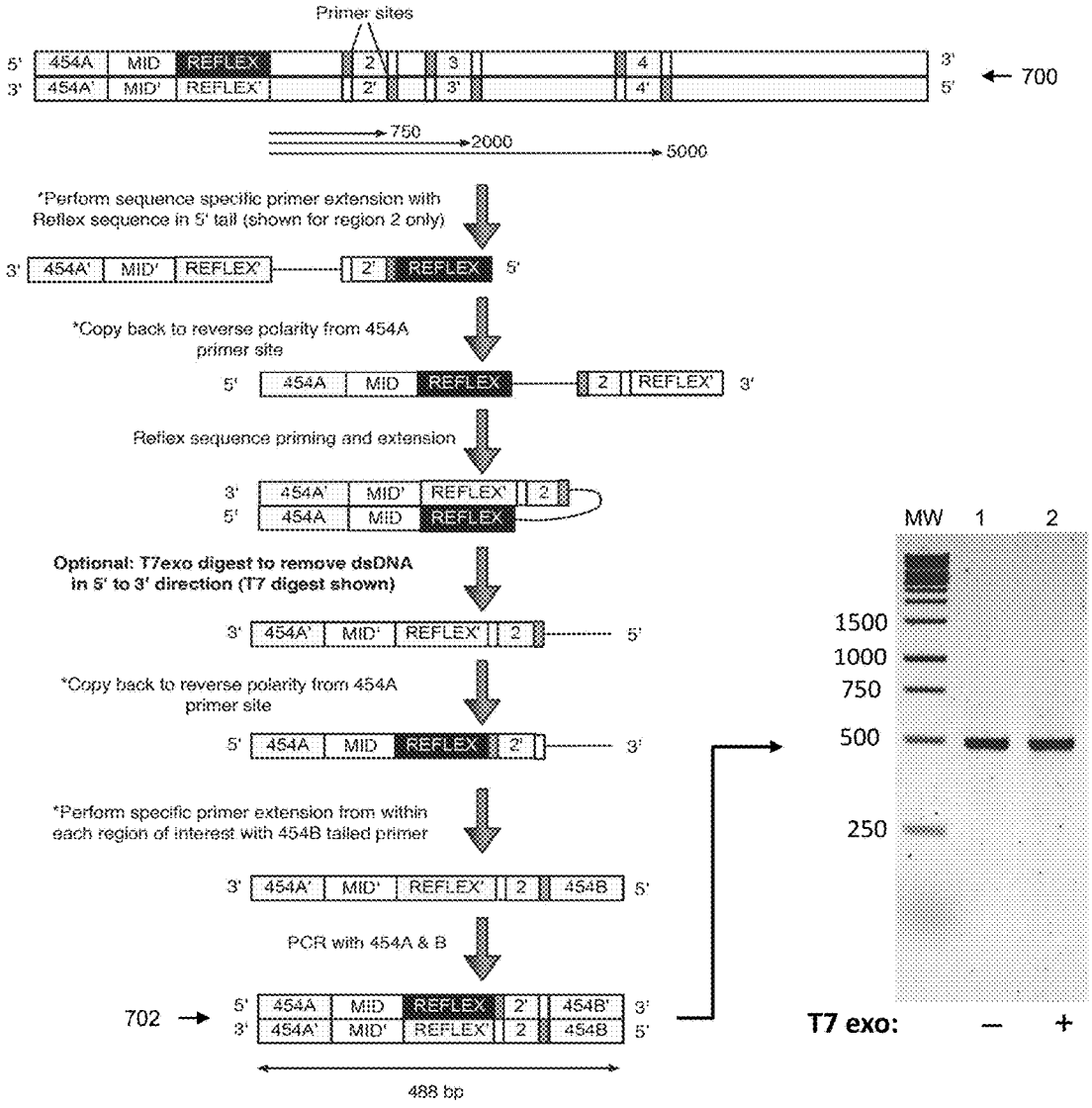

no enzyme control
[Taq]
no taq control
[Taq]
+ T7 exo
[Taq]
+ competitor
130
100
82
1 2 3 4 5 6 7 8 9 10 11 12 13 14

Fig. 7



Primer extension reactions with * may be performed such that isolation of single strand species is facilitated (e.g., using primers with binding moieties and/or multiple cycles of extension)

Fig. 8

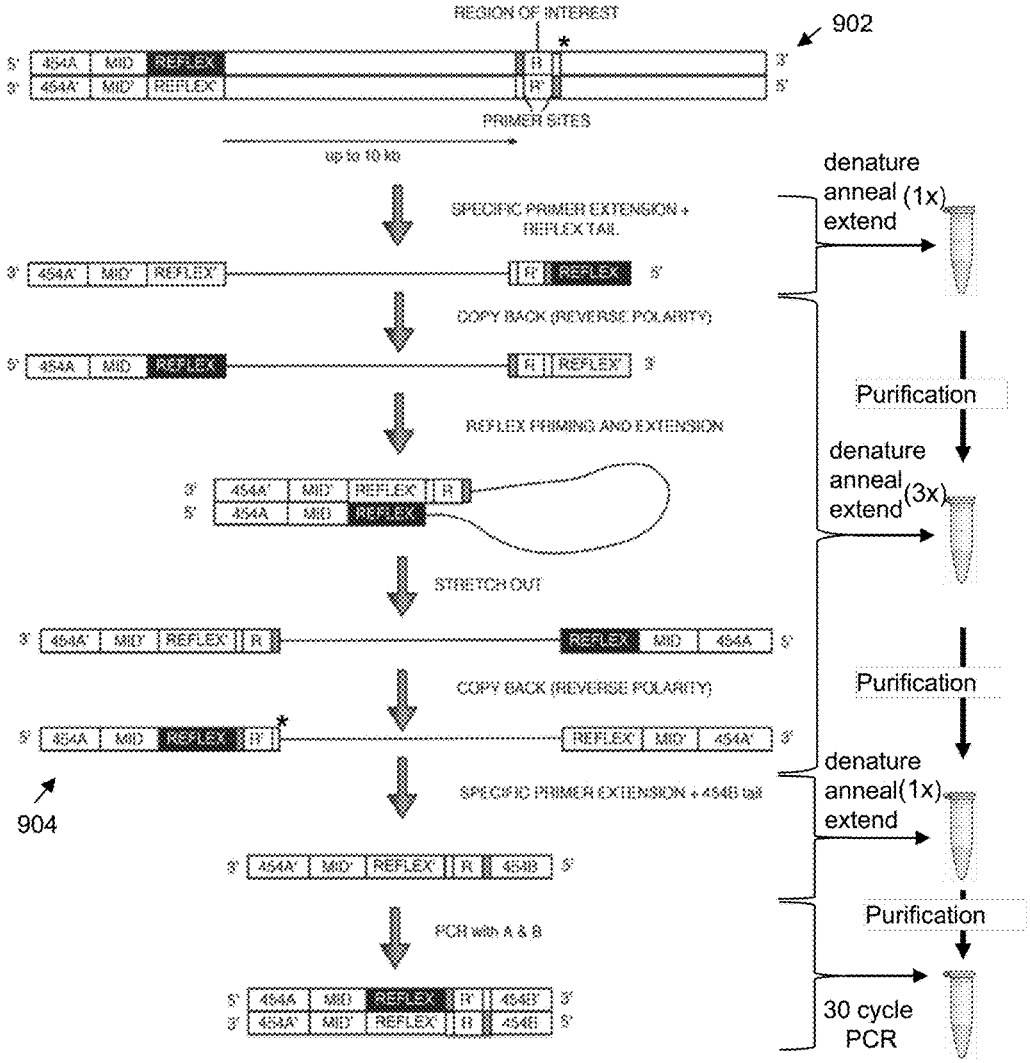

REGION OF INTEREST
902
PRIMER SITES
up to 10 kb
SPECIFIC PRIMER EXTENSION + REFLEX TAIL
COPY BACK (REVERSE POLARITY)
REFLEX PRIMING AND EXTENSION
STRETCH OUT
COPY BACK (REVERSE POLARITY)
SPECIFIC PRIMER EXTENSION + 454B tail
904
PCR with A & B
denature
anneal (1x)
extend
Purification
denature
anneal
extend (3x)
Purification
denature
anneal(1x)
extend
Purification
30 cycle
PCR

Fig. 9

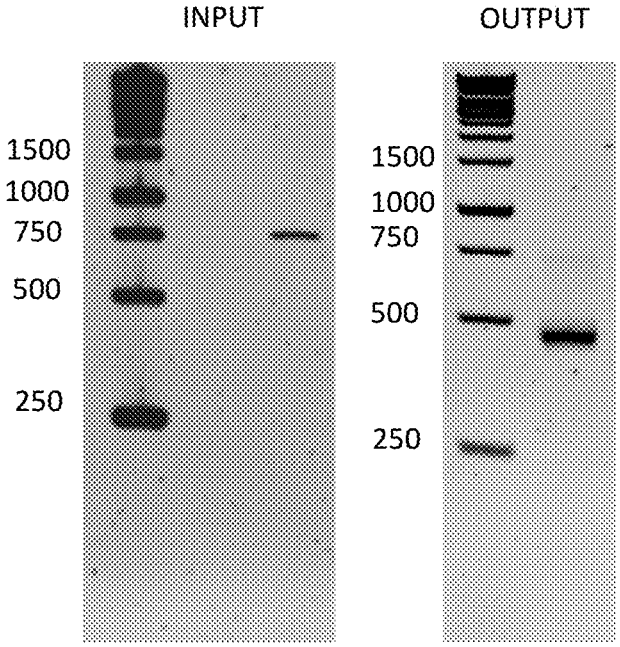


Fig. 10

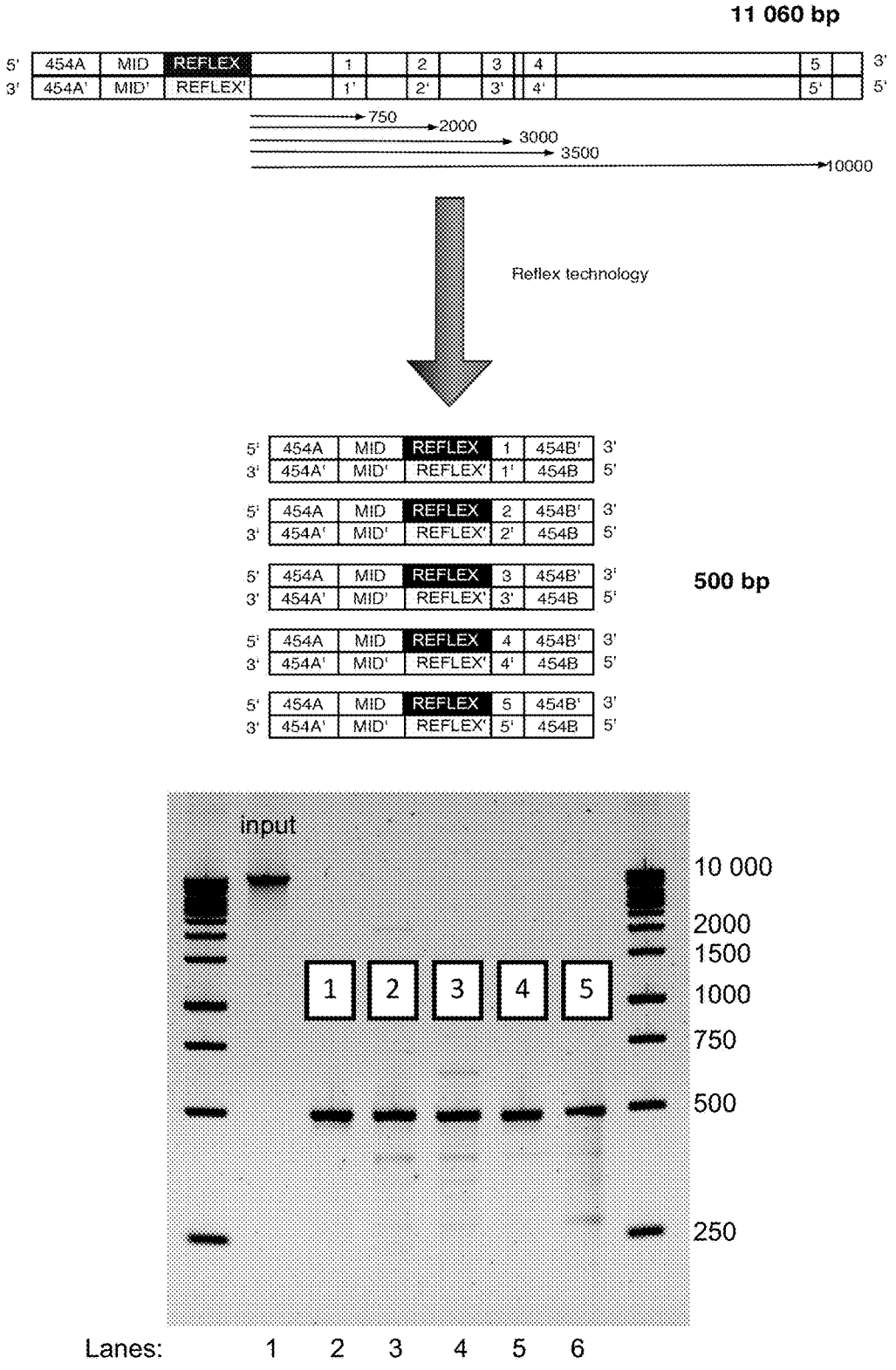
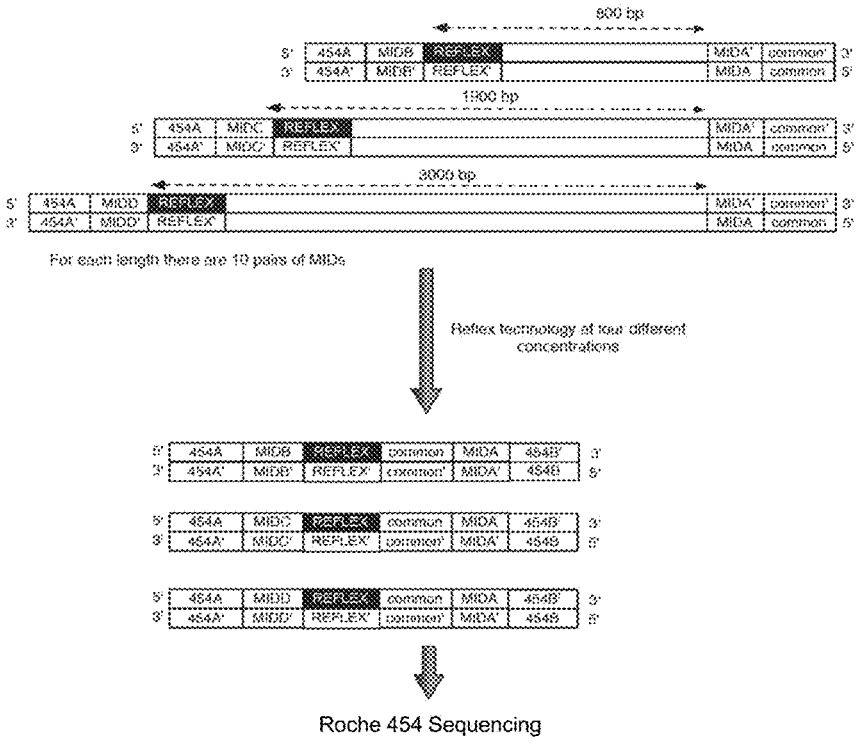

11 060 bp
5' 454A MID REFLEX 1 2 3 4 5 3'
3' 454A' MID' REFLEX' 1' 2' 3' 4' 5' 5'
750
2000
3000
3500
10000
Reflex technology
5' 454A MID REFLEX 1 454B' 3'
3' 454A' MID' REFLEX' 1' 454B 5'
5' 454A MID REFLEX 2 454B' 3'
3' 454A' MID' REFLEX' 2' 454B 5'
5' 454A MID REFLEX 3 454B' 3'
3' 454A' MID' REFLEX' 3' 454B 5'
500 bp
5' 454A MID REFLEX 4 454B' 3'
3' 454A' MID' REFLEX' 4' 454B 5'
5' 454A MID REFLEX 5 454B' 3'
3' 454A' MID' REFLEX' 5' 454B 5'
input
10 000
2000
1500
1000
750
500
250
1
2
3
4
5
Lanes: 1 2 3 4 5 6

Fig. 11


800 bp
1900 bp
3000 bp
454A
MIDB
REFLEX
MIDA
common
454B
For each length there are 10 pairs of MIDs
Reflex technology at four different concentrations
Roche 454 Sequencing

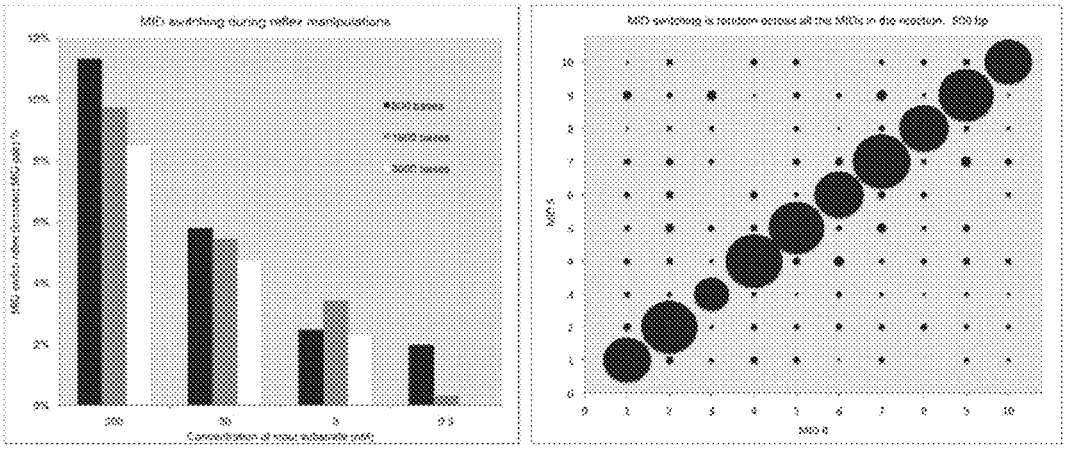

MID switching during reflex manipulations
MID switching is random across all the MIDs in the reaction. 800 bp

Fig. 12

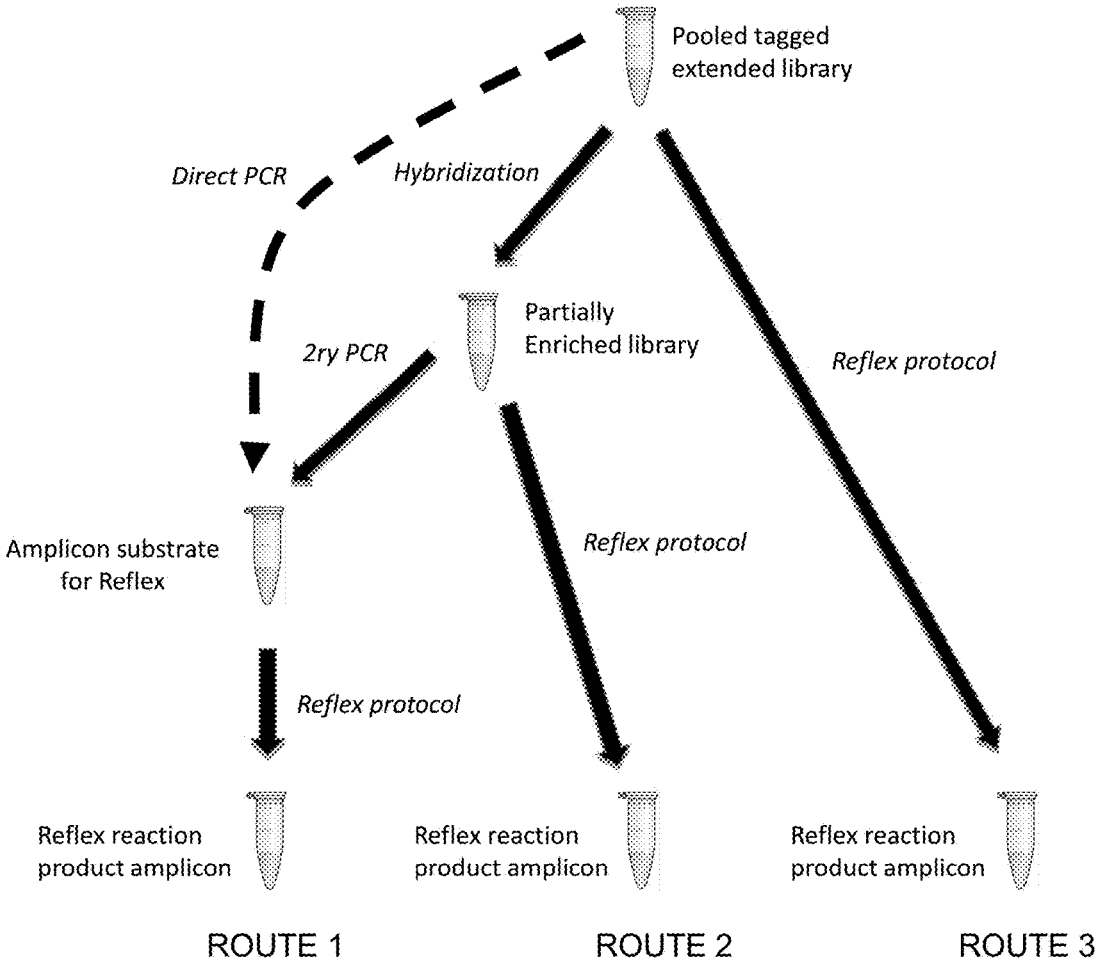

Pooled tagged extended library
Direct PCR
Hybridization
Partially Enriched library
2ry PCR
Reflex protocol
Amplicon substrate for Reflex
Reflex protocol
Reflex protocol
Reflex reaction product amplicon
Reflex reaction product amplicon
Reflex reaction product amplicon
ROUTE 1
ROUTE 2
ROUTE 3

Fig. 13

METHODS FOR ANALYZING NUCLEIC ACIDS FROM SINGLE CELLS

CROSS REFERENCE TO RELATED APPLICATION

This application is continuation of U.S. application Ser. No. 14/792,094, filed Jul. 6, 2015, which is a continuation of U.S. application Ser. No. 14/172,694, filed Feb. 4, 2014, now U.S. Pat. No. 9,102,980, which is a continuation of U.S. application Ser. No. 14/021,790, filed Sep. 9, 2013, now U.S. Pat. No. 8,679,756, which is a continuation of U.S. application Ser. No. 13/859,450, filed Apr. 9, 2013, now U.S. Pat. No. 8,563,274, which is a continuation of U.S. application Ser. No. 13/622,872, filed Sep. 19, 2012, which is a continuation of U.S. application Ser. No. 13/387,343, filed Feb. 15, 2012, now U.S. Pat. No. 8,298,767, which is a § 371 National Phase Application of PCT/IB2010/002243, filed Aug. 13, 2010, which claims priority to U.S. Provisional Application No. 61/235,595, filed Aug. 20, 2009 and U.S. Provisional Application No. 61/288,792, filed Dec. 21, 2009; all of which are herein incorporated by reference.

BACKGROUND OF THE INVENTION

We have previously described methods that enable tagging each of a population of fragmented genomes and then combining them together to create a 'population library' that can be processed and eventually sequenced as a mixture. The population tags enable analysis software to parse the sequence reads into files that can be attributed to a particular genome in the population. One limitation of the overall process stems from limitations of existing DNA sequencing technologies. In particular, if fragments in the regions of interest of the genome are longer than the lengths that can be sequenced by a particular technology, then such fragments will not be fully analyzed (since sequencing proceeds from an end of a fragment inward). Furthermore, a disadvantage of any sequencing technology dependent on fragmentation is that sequence changes in one part of a particular genomic region may not be able to be linked to sequence changes in other parts of the same genome (e.g., the same chromosome) because the sequence changes reside on different fragments. (See FIG. **5** and its description below).

The present invention removes the limitations imposed by current sequencing technologies as well as being useful in a number of other nucleic acid analyses.

SUMMARY OF THE INVENTION

Aspects of the present invention are drawn to processes for moving a region of interest in a polynucleotide from a first position to a second position with regard to a domain within the polynucleotide, also referred to as a "reflex method" (or reflex process, reflex sequence process, reflex reaction, and the like). In certain embodiments, the reflex method results in moving a region of interest into functional proximity to specific domain elements present in the polynucleotide (e.g., primer sites and/or MID). Compositions, kits and systems that find use in carrying out the reflex processes described herein are also provided.

BRIEF DESCRIPTION OF THE DRAWINGS

The invention is best understood from the following detailed description when read in conjunction with the accompanying drawings. It is emphasized that, according to common practice, the various features of the drawings are not to scale. Indeed, the dimensions of the various features are arbitrarily expanded or reduced for clarity. Included in the drawings are the following figures:

FIG. **1**: Panel A is a schematic diagram illustrating moving a first domain from one site to another in a nucleic acid molecule using a reflex sequence. Panel B is a schematic diagram depicting the relative position of primer pairs (A_n-B_n primers) that find use in aspects of the reflex process described herein.

FIG. **2** shows an exemplary embodiment of using binding partner pairs (biotin/streptavidin) to isolate single stranded polynucleotides of interest.

FIG. **3** is a schematic diagram illustrating an exemplary embodiment for moving a primer site and a MID to a specific location in a nucleic acid of interest.

FIG. **4** shows a schematic diagram illustrating an exemplary use of the reflex process for generating a sample enriched for fragments having a region of interest (e.g., from a population of randomly fragmented and asymmetrically tagged polynucleotides).

FIG. **5** shows a comparison of methods for identifying nucleic acid polymorphisms in homologous nucleic acids in a sample (e.g., the same region derived from a chromosomal pair of a diploid cell or viral genomes/transcripts). The top schematic shows two nucleic acid molecules in a sample (**1** and **2**) having a different assortment of polymorphisms in polymorphic sites A, B and C (A**1**, B**1**, C**1** and C**2**). Standard sequencing methods using fragmentation (left side) can identify the polymorphisms in these nucleic acids but do not retain linkage information. Employing the reflex process described herein to identify polymorphisms (right side) maintains linkage information.

FIG. **6**: Panel A is a schematic showing expected structures and sizes of nucleic acid species in the reflex process; Panel B is a polyacrylamide gel showing the nucleic acid species produced in the reflex process described in Example 1.

FIG. **7**: Panel A is a schematic showing the structure of the nucleic acid and competitor used in the reflex process; Panel B is a polyacrylamide gel showing the nucleic acid species produced in the reflex process described in Example 1.

FIG. **8** shows a flow chart of a reflex process (left) in which the T7 exonuclease step is optional. The gel on the right shows the resultant product of the reflex process either without the T7 exonuclease step (lane 1) or with the T7 exonuclease step (lane 2).

FIG. **9** shows an exemplary reflex process workflow with indications on the right as to where purification of reaction products is employed (e.g., using Agencourt beads to remove primer oligos).

FIG. **10** shows the starting material (left panel) and the resultant product generated (right panel) using a reflex process without using a T7 exonuclease step (as described in Example II). The reflex site in the starting material is a sequence normally present in the polynucleotide being processed (also called a "non-artificial" reflex site). This figure shows that the 755 base pair starting nucleic acid was processed to the expected 461 base pair product, thus confirming that a "non-artificial" reflex site is effective in transferring an adapter domain from one location to another in a polynucleotide of interest in a sequence specific manner.

FIG. **11** shows a schematic and results of an experiment in which the reflex process is performed on a single large initial template (a "parent" fragment) to generate five different products ("daughter" products) each having a differ-

ent region of interest (i.e., daughter products are produced having either region **1**, **2**, **3**, **4** or **5**).

FIG. **12** shows a schematic and results of experiments performed to determine the prevalence of intramolecular rearrangement during the reflex process (as desired) vs. intermolecular rearrangement (MID switching).

FIG. **13** shows a diagram of exemplary workflows for preparing material for and performing the reflex process.

DEFINITIONS

Unless otherwise defined, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. Still, certain elements are defined for the sake of clarity and ease of reference.

Terms and symbols of nucleic acid chemistry, biochemistry, genetics, and molecular biology used herein follow those of standard treatises and texts in the field, e.g. Kornberg and Baker, DNA Replication, Second Edition (W.H. Freeman, New York, 1992); Lehninger, Biochemistry, Second Edition (Worth Publishers, New York, 1975); Strachan and Read, Human Molecular Genetics, Second Edition (Wiley-Liss, New York, 1999); Eckstein, editor, Oligonucleotides and Analogs: A Practical Approach (Oxford University Press, New York, 1991); Gait, editor, Oligonucleotide Synthesis: A Practical Approach (IRL Press, Oxford, 1984); and the like.

"Amplicon" means the product of a polynucleotide amplification reaction. That is, it is a population of polynucleotides, usually double stranded, that are replicated from one or more starting sequences. The one or more starting sequences may be one or more copies of the same sequence, or it may be a mixture of different sequences. Amplicons may be produced by a variety of amplification reactions whose products are multiple replicates of one or more target nucleic acids. Generally, amplification reactions producing amplicons are "template-driven" in that base pairing of reactants, either nucleotides or oligonucleotides, have complements in a template polynucleotide that are required for the creation of reaction products. In one aspect, template-driven reactions are primer extensions with a nucleic acid polymerase or oligonucleotide ligations with a nucleic acid ligase. Such reactions include, but are not limited to, polymerase chain reactions (PCRs), linear polymerase reactions, nucleic acid sequence-based amplification (NASBAs), rolling circle amplifications, and the like, disclosed in the following references that are incorporated herein by reference: Mullis et al, U.S. Pat. Nos. 4,683,195; 4,965,188; 4,683,202; 4,800,159 (PCR); Gelfand et al, U.S. Pat. No. 5,210,015 (real-time PCR with "TAQMAN™" probes); Wittwer et al, U.S. Pat. No. 6,174,670; Kacian et al, U.S. Pat. No. 5,399,491 ("NASBA"); Lizardi, U.S. Pat. No. 5,854,033; Aono et al, Japanese patent publ. JP 4-262799 (rolling circle amplification); and the like. In one aspect, amplicons of the invention are produced by PCRs. An amplification reaction may be a "real-time" amplification if a detection chemistry is available that permits a reaction product to be measured as the amplification reaction progresses, e.g. "real-time PCR" described below, or "real-time NASBA" as described in Leone et al, Nucleic Acids Research, 26: 2150-2155 (1998), and like references. As used herein, the term "amplifying" means performing an amplification reaction. A "reaction mixture" means a solution containing all the necessary reactants for performing a reaction, which may include, but not be limited to, buffering agents to maintain pH at a selected level during a reaction, salts, co-factors, scavengers, and the like.

The term "assessing" includes any form of measurement, and includes determining if an element is present or not. The terms "determining", "measuring", "evaluating", "assessing" and "assaying" are used interchangeably and includes quantitative and qualitative determinations. Assessing may be relative or absolute. "Assessing the presence of" includes determining the amount of something present, and/or determining whether it is present or absent. As used herein, the terms "determining," "measuring," and "assessing," and "assaying" are used interchangeably and include both quantitative and qualitative determinations.

Polynucleotides that are "asymmetrically tagged" have left and right adapter domains that are not identical. This process is referred to generically as attaching adapters asymmetrically or asymmetrically tagging a polynucleotide, e.g., a polynucleotide fragment. Production of polynucleotides having asymmetric adapter termini may be achieved in any convenient manner. Exemplary asymmetric adapters are described in: U.S. Pat. Nos. 5,712,126 and 6,372,434; U.S. Patent Publications 2007/0128624 and 2007/0172839; and PCT publication WO/2009/032167; all of which are incorporated by reference herein in their entirety. In certain embodiments, the asymmetric adapters employed are those described in U.S. patent application Ser. No. 12/432,080, filed on Apr. 29, 2009, incorporated herein by reference in its entirety.

As one example, a user of the subject invention may use an asymmetric adapter to tag polynucleotides. An "asymmetric adapter" is one that, when ligated to both ends of a double stranded nucleic acid fragment, will lead to the production of primer extension or amplification products that have non-identical sequences flanking the genomic insert of interest. The ligation is usually followed by subsequent processing steps so as to generate the non-identical terminal adapter sequences. For example, replication of an asymmetric adapter attached fragment(s) results in polynucleotide products in which there is at least one nucleic acid sequence difference, or nucleotide/nucleoside modification, between the terminal adapter sequences. Attaching adapters asymmetrically to polynucleotides (e.g., polynucleotide fragments) results in polynucleotides that have one or more adapter sequences on one end (e.g., one or more region or domain, e.g., a primer site) that are either not present or have a different nucleic acid sequence as compared to the adapter sequence on the other end. It is noted that an adapter that is termed an "asymmetric adapter" is not necessarily itself structurally asymmetric, nor does the mere act of attaching an asymmetric adapter to a polynucleotide fragment render it immediately asymmetric. Rather, an asymmetric adapter-attached polynucleotide, which has an identical asymmetric adapter at each end, produces replication products (or isolated single stranded polynucleotides) that are asymmetric with respect to the adapter sequences on opposite ends (e.g., after at least one round of amplification/primer extension).

Any convenient asymmetric adapter, or process for attaching adapters asymmetrically, may be employed in practicing the present invention. Exemplary asymmetric adapters are described in: U.S. Pat. Nos. 5,712,126 and 6,372,434; U.S. Patent Publications 2007/0128624 and 2007/0172839; and PCT publication WO/2009/032167; all of which are incorporated by reference herein in their entirety. In certain embodiments, the asymmetric adapters

employed are those described in U.S. patent application Ser. No. 12/432,080, filed on Apr. 29, 2009, incorporated herein by reference in its entirety.

"Complementary" or "substantially complementary" refers to the hybridization or base pairing or the formation of a duplex between nucleotides or nucleic acids, such as, for instance, between the two strands of a double stranded DNA molecule or between an oligonucleotide primer and a primer site on a single stranded nucleic acid. Complementary nucleotides are, generally, A and T (or A and U), or C and G. Two single stranded RNA or DNA molecules are said to be substantially complementary when the nucleotides of one strand, optimally aligned and compared and with appropriate nucleotide insertions or deletions, pair with at least about 80% of the nucleotides of the other strand, usually at least about 90% to 95%, and more preferably from about 98 to 100%. Alternatively, substantial complementarity exists when an RNA or DNA strand will hybridize under selective hybridization conditions to its complement. Typically, selective hybridization will occur when there is at least about 65% complementary over a stretch of at least 14 to 25 nucleotides, preferably at least about 75%, more preferably at least about 90% complementary. See, M. Kanehisa Nucleic Acids Res. 12:203 (1984), incorporated herein by reference.

"Duplex" means at least two oligonucleotides and/or polynucleotides that are fully or partially complementary undergo Watson-Crick type base pairing among all or most of their nucleotides so that a stable complex is formed. The terms "annealing" and "hybridization" are used interchangeably to mean the formation of a stable duplex. "Perfectly matched" in reference to a duplex means that the poly- or oligonucleotide strands making up the duplex form a double stranded structure with one another such that every nucleotide in each strand undergoes Watson-Crick base pairing with a nucleotide in the other strand. A stable duplex can include Watson-Crick base pairing and/or non-Watson-Crick base pairing between the strands of the duplex (where base pairing means the forming hydrogen bonds). In certain embodiments, a non-Watson-Crick base pair includes a nucleoside analog, such as deoxyinosine, 2,6-diaminopurine, PNAs, LNA's and the like. In certain embodiments, a non-Watson-Crick base pair includes a "wobble base", such as deoxyinosine, 8-oxo-dA, 8-oxo-dG and the like, where by "wobble base" is meant a nucleic acid base that can base pair with a first nucleotide base in a complementary nucleic acid strand but that, when employed as a template strand for nucleic acid synthesis, leads to the incorporation of a second, different nucleotide base into the synthesizing strand (wobble bases are described in further detail below). A "mismatch" in a duplex between two oligonucleotides or polynucleotides means that a pair of nucleotides in the duplex fails to undergo Watson-Crick bonding.

"Genetic locus," "locus," or "locus of interest" in reference to a genome or target polynucleotide, means a contiguous sub-region or segment of the genome or target polynucleotide. As used herein, genetic locus, locus, or locus of interest may refer to the position of a nucleotide, a gene or a portion of a gene in a genome, including mitochondrial DNA or other non-chromosomal DNA (e.g., bacterial plasmid), or it may refer to any contiguous portion of genomic sequence whether or not it is within, or associated with, a gene. A genetic locus, locus, or locus of interest can be from a single nucleotide to a segment of a few hundred or a few thousand nucleotides in length or more. In general, a locus of interest will have a reference sequence associated with it (see description of "reference sequence" below).

"Kit" refers to any delivery system for delivering materials or reagents for carrying out a method of the invention. In the context of reaction assays, such delivery systems include systems that allow for the storage, transport, or delivery of reaction reagents (e.g., probes, enzymes, etc. in the appropriate containers) and/or supporting materials (e.g., buffers, written instructions for performing the assay etc.) from one location to another. For example, kits include one or more enclosures (e.g., boxes) containing the relevant reaction reagents and/or supporting materials. Such contents may be delivered to the intended recipient together or separately. For example, a first container may contain an enzyme for use in an assay, while a second container contains probes.

"Ligation" means to form a covalent bond or linkage between the termini of two or more nucleic acids, e.g. oligonucleotides and/or polynucleotides, in a template-driven reaction. The nature of the bond or linkage may vary widely and the ligation may be carried out enzymatically or chemically. As used herein, ligations are usually carried out enzymatically to form a phosphodiester linkage between a 5' carbon of a terminal nucleotide of one oligonucleotide with 3' carbon of another oligonucleotide. A variety of template-driven ligation reactions are described in the following references, which are incorporated by reference: Whiteley et al, U.S. Pat. No. 4,883,750; Letsinger et al, U.S. Pat. No. 5,476,930; Fung et al, U.S. Pat. No. 5,593,826; Kool, U.S. Pat. No. 5,426,180; Landegren et al, U.S. Pat. No. 5,871,921; Xu and Kool, Nucleic Acids Research, 27: 875-881 (1999); Higgins et al, Methods in Enzymology, 68: 50-71 (1979); Engler et al, The Enzymes, 15: 3-29 (1982); and Namsaraev, U.S. patent publication 2004/0110213.

"Multiplex Identifier" (MID) as used herein refers to a tag or combination of tags associated with a polynucleotide whose identity (e.g., the tag DNA sequence) can be used to differentiate polynucleotides in a sample. In certain embodiments, the MID on a polynucleotide is used to identify the source from which the polynucleotide is derived. For example, a nucleic acid sample may be a pool of polynucleotides derived from different sources, (e.g., polynucleotides derived from different individuals, different tissues or cells, or polynucleotides isolated at different times points), where the polynucleotides from each different source are tagged with a unique MID. As such, a MID provides a correlation between a polynucleotide and its source. In certain embodiments, MIDs are employed to uniquely tag each individual polynucleotide in a sample. Identification of the number of unique MIDs in a sample can provide a readout of how many individual polynucleotides are present in the sample (or from how many original polynucleotides a manipulated polynucleotide sample was derived; see, e.g., U.S. Pat. No. 7,537,897, issued on May 26, 2009, incorporated herein by reference in its entirety). MIDs can range in length from 2 to 100 nucleotide bases or more and may include multiple subunits, where each different MID has a distinct identity and/or order of subunits. Exemplary nucleic acid tags that find use as MIDs are described in U.S. Pat. No. 7,544,473, issued on Jun. 6, 2009, and titled "Nucleic Acid Analysis Using Sequence Tokens", as well as U.S. Pat. No. 7,393,665, issued on Jul. 1, 2008, and titled "Methods and Compositions for Tagging and Identifying Polynucleotides", both of which are incorporated herein by reference in their entirety for their description of nucleic acid tags and their use in identifying polynucleotides. In certain embodiments, a set of MIDs employed to tag a plurality of samples need not have any particular common property (e.g., Tm, length, base composition, etc.), as the methods described herein can

accommodate a wide variety of unique MID sets. It is emphasized here that MIDs need only be unique within a given experiment. Thus, the same MID may be used to tag a different sample being processed in a different experiment. In addition, in certain experiments, a user may use the same MID to tag a subset of different samples within the same experiment. For example, all samples derived from individuals having a specific phenotype may be tagged with the same MID, e.g., all samples derived from control (or wild-type) subjects can be tagged with a first MID while subjects having a disease condition can be tagged with a second MID (different than the first MID). As another example, it may be desirable to tag different samples derived from the same source with different MIDs (e.g., samples derived over time or derived from different sites within a tissue). Further, MIDs can be generated in a variety of different ways, e.g., by a combinatorial tagging approach in which one MID is attached by ligation and a second MID is attached by primer extension. Thus, MIDs can be designed and implemented in a variety of different ways to track polynucleotide fragments during processing and analysis, and thus no limitation in this regard is intended.

"Nucleoside" as used herein includes the natural nucleosides, including 2'-deoxy and 2'-hydroxyl forms, e.g. as described in Kornberg and Baker, DNA Replication, 2nd Ed. (Freeman, San Francisco, 1992). "Analogs" in reference to nucleosides includes synthetic nucleosides having modified base moieties and/or modified sugar moieties, e.g. described by Scheit, Nucleotide Analogs (John Wiley, New York, 1980); Uhlman and Peyman, Chemical Reviews, 90: 543-584 (1990), or the like, with the proviso that they are capable of specific hybridization. Such analogs include synthetic nucleosides designed to enhance binding properties, reduce complexity, increase specificity, and the like. Polynucleotides comprising analogs with enhanced hybridization or nuclease resistance properties are described in Uhlman and Peyman (cited above); Crooke et al, Exp. Opin. Ther. Patents, 6: 855-870 (1996); Mesmaeker et al, Current Opinion in Structual Biology, 5: 343-355 (1995); and the like. Exemplary types of polynucleotides that are capable of enhancing duplex stability include oligonucleotide phosphoramidates (referred to herein as "amidates"), peptide nucleic acids (referred to herein as "PNAs"), oligo-2'-O-alkylribonucleotides, polynucleotides containing C-5 propynylpyrimidines, locked nucleic acids ("LNAs"), and like compounds. Such oligonucleotides are either available commercially or may be synthesized using methods described in the literature.

"Polymerase chain reaction," or "PCR," means a reaction for the in vitro amplification of specific DNA sequences by the simultaneous primer extension of complementary strands of DNA. In other words, PCR is a reaction for making multiple copies or replicates of a target nucleic acid flanked by primer sites, such reaction comprising one or more repetitions of the following steps: (i) denaturing the target nucleic acid, (ii) annealing primers to the primer sites, and (iii) extending the primers by a nucleic acid polymerase in the presence of nucleoside triphosphates. Usually, the reaction is cycled through different temperatures optimized for each step in a thermal cycler instrument. Particular temperatures, durations at each step, and rates of change between steps depend on many factors well-known to those of ordinary skill in the art, e.g. exemplified by the references: McPherson et al, editors, PCR: A Practical Approach and PCR2: A Practical Approach (IRL Press, Oxford, 1991 and 1995, respectively). For example, in a conventional PCR using Taq DNA polymerase, a double stranded target nucleic acid may be denatured at a temperature >90° C., primers annealed at a temperature in the range 50-75° C., and primers extended at a temperature in the range 72-78° C. The term "PCR" encompasses derivative forms of the reaction, including but not limited to, RT-PCR, real-time PCR, nested PCR, quantitative PCR, multiplexed PCR, and the like. Reaction volumes range from a few hundred nanoliters, e.g. 200 nL, to a few hundred μL, e.g. 200 μL. "Reverse transcription PCR," or "RT-PCR," means a PCR that is preceded by a reverse transcription reaction that converts a target RNA to a complementary single stranded DNA, which is then amplified, e.g. Tecott et al, U.S. Pat. No. 5,168,038, which patent is incorporated herein by reference. "Real-time PCR" means a PCR for which the amount of reaction product, i.e. amplicon, is monitored as the reaction proceeds. There are many forms of real-time PCR that differ mainly in the detection chemistries used for monitoring the reaction product, e.g. Gelfand et al, U.S. Pat. No. 5,210,015 ("TAQMAN™"); Wittwer et al, U.S. Pat. Nos. 6,174,670 and 6,569,627 (intercalating dyes); Tyagi et al, U.S. Pat. No. 5,925,517 (molecular beacons); which patents are incorporated herein by reference. Detection chemistries for real-time PCR are reviewed in Mackay et al, Nucleic Acids Research, 30: 1292-1305 (2002), which is also incorporated herein by reference. "Nested PCR" means a two-stage PCR wherein the amplicon of a first PCR becomes the sample for a second PCR using a new set of primers, at least one of which binds to an interior location of the first amplicon. As used herein, "initial primers" in reference to a nested amplification reaction mean the primers used to generate a first amplicon, and "secondary primers" mean the one or more primers used to generate a second, or nested, amplicon. "Multiplexed PCR" means a PCR wherein multiple target sequences (or a single target sequence and one or more reference sequences) are simultaneously carried out in the same reaction mixture, e.g. Bernard et al, Anal. Biochem., 273: 221-228 (1999)(two-color real-time PCR). Usually, distinct sets of primers are employed for each sequence being amplified.

"Quantitative PCR" means a PCR designed to measure the abundance of one or more specific target sequences in a sample or specimen. Quantitative PCR includes both absolute quantitation and relative quantitation of such target sequences. Quantitative measurements are made using one or more reference sequences that may be assayed separately or together with a target sequence. The reference sequence may be endogenous or exogenous to a sample or specimen, and in the latter case, may comprise one or more competitor templates. Typical endogenous reference sequences include segments of transcripts of the following genes: β-actin, GAPDH, β_2-microglobulin, ribosomal RNA, and the like. Techniques for quantitative PCR are well-known to those of ordinary skill in the art, as exemplified in the following references that are incorporated by reference: Freeman et al, Biotechniques, 26: 112-126 (1999); Becker-Andre et al, Nucleic Acids Research, 17: 9437-9447 (1989); Zimmerman et al, Biotechniques, 21: 268-279 (1996); Diviacco et al, Gene, 122: 3013-3020 (1992); Becker-Andre et al, Nucleic Acids Research, 17: 9437-9446 (1989); and the like.

"Polynucleotide" or "oligonucleotide" is used interchangeably and each means a linear polymer of nucleotide monomers. Monomers making up polynucleotides and oligonucleotides are capable of specifically binding to a natural polynucleotide by way of a regular pattern of monomer-to-monomer interactions, such as Watson-Crick type of base pairing, base stacking, Hoogsteen or reverse Hoogsteen types of base pairing, wobble base pairing, or the like. As

described in detail below, by "wobble base" is meant a nucleic acid base that can base pair with a first nucleotide base in a complementary nucleic acid strand but that, when employed as a template strand for nucleic acid synthesis, leads to the incorporation of a second, different nucleotide base into the synthesizing strand. Such monomers and their internucleosidic linkages may be naturally occurring or may be analogs thereof, e.g. naturally occurring or non-naturally occurring analogs. Non-naturally occurring analogs may include peptide nucleic acids (PNAs, e.g., as described in U.S. Pat. No. 5,539,082, incorporated herein by reference), locked nucleic acids (LNAs, e.g., as described in U.S. Pat. No. 6,670,461, incorporated herein by reference), phosphorothioate internucleosidic linkages, bases containing linking groups permitting the attachment of labels, such as fluorophores, or haptens, and the like. Whenever the use of an oligonucleotide or polynucleotide requires enzymatic processing, such as extension by a polymerase, ligation by a ligase, or the like, one of ordinary skill would understand that oligonucleotides or polynucleotides in those instances would not contain certain analogs of internucleosidic linkages, sugar moieties, or bases at any or some positions. Polynucleotides typically range in size from a few monomeric units, e.g. 5-40, when they are usually referred to as "oligonucleotides," to several thousand monomeric units. Whenever a polynucleotide or oligonucleotide is represented by a sequence of letters (upper or lower case), such as "ATGCCTG," it will be understood that the nucleotides are in 5'->3' order from left to right and that "A" denotes deoxyadenosine, "C" denotes deoxycytidine, "G" denotes deoxyguanosine, and "T" denotes thymidine, "I" denotes deoxyinosine, "U" denotes uridine, unless otherwise indicated or obvious from context. Unless otherwise noted the terminology and atom numbering conventions will follow those disclosed in Strachan and Read, Human Molecular Genetics 2 (Wiley-Liss, New York, 1999). Usually polynucleotides comprise the four natural nucleosides (e.g. deoxyadenosine, deoxycytidine, deoxyguanosine, deoxythymidine for DNA or their ribose counterparts for RNA) linked by phosphodiester linkages; however, they may also comprise non-natural nucleotide analogs, e.g. including modified bases, sugars, or internucleosidic linkages. It is clear to those skilled in the art that where an enzyme has specific oligonucleotide or polynucleotide substrate requirements for activity, e.g. single stranded DNA, RNA/DNA duplex, or the like, then selection of appropriate composition for the oligonucleotide or polynucleotide substrates is well within the knowledge of one of ordinary skill, especially with guidance from treatises, such as Sambrook et al, Molecular Cloning, Second Edition (Cold Spring Harbor Laboratory, New York, 1989), and like references.

"Primer" means an oligonucleotide, either natural or synthetic, that is capable, upon forming a duplex with a polynucleotide template, of acting as a point of initiation of nucleic acid synthesis and being extended from its 3' end along the template so that an extended duplex is formed. The sequence of nucleotides added during the extension process is determined by the sequence of the template polynucleotide. Usually primers are extended by a DNA polymerase. Primers are generally of a length compatible with their use in synthesis of primer extension products, and are usually are in the range of between 8 to 100 nucleotides in length, such as 10 to 75, 15 to 60, 15 to 40, 18 to 30, 20 to 40, 21 to 50, 22 to 45, 25 to 40, and so on, more typically in the range of between 18-40, 20-35, 21-30 nucleotides long, and any length between the stated ranges. Typical primers can be in the range of between 10-50 nucleotides long, such as 15-45, 18-40, 20-30, 21-25 and so on, and any length between the stated ranges. In some embodiments, the primers are usually not more than about 10, 12, 15, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 35, 40, 45, 50, 55, 60, 65, or 70 nucleotides in length.

Primers are usually single-stranded for maximum efficiency in amplification, but may alternatively be double-stranded. If double-stranded, the primer is usually first treated to separate its strands before being used to prepare extension products. This denaturation step is typically affected by heat, but may alternatively be carried out using alkali, followed by neutralization. Thus, a "primer" is complementary to a template, and complexes by hydrogen bonding or hybridization with the template to give a primer/template complex for initiation of synthesis by a polymerase, which is extended by the addition of covalently bonded bases linked at its 3' end complementary to the template in the process of DNA synthesis.

A "primer pair" as used herein refers to first and second primers having nucleic acid sequence suitable for nucleic acid-based amplification of a target nucleic acid. Such primer pairs generally include a first primer having a sequence that is the same or similar to that of a first portion of a target nucleic acid, and a second primer having a sequence that is complementary to a second portion of a target nucleic acid to provide for amplification of the target nucleic acid or a fragment thereof. Reference to "first" and "second" primers herein is arbitrary, unless specifically indicated otherwise. For example, the first primer can be designed as a "forward primer" (which initiates nucleic acid synthesis from a 5' end of the target nucleic acid) or as a "reverse primer" (which initiates nucleic acid synthesis from a 5' end of the extension product produced from synthesis initiated from the forward primer). Likewise, the second primer can be designed as a forward primer or a reverse primer.

"Primer site" (e.g., a sequencing primer site, and amplification primer site, etc.) as used herein refers to a domain in a polynucleotide that includes the sequence of a primer (e.g., a sequencing primer) and/or the complementary sequence of a primer. When present in single stranded form (e.g., in a single stranded polynucleotide), a primer site can be either the identical sequence of a primer or the complementary sequence of a primer. When present in double stranded form, a primer site contains the sequence of a primer hybridized to the complementary sequence of the primer. Thus, a primer site is a region of a polynucleotide that is either identical to or complementary to the sequence of a primer (when in a single stranded form) or a double stranded region formed between a primer sequence and its complement. Primer sites may be present in an adapter attached to a polynucleotide. The specific orientation of a primer site can be inferred by those of ordinary skill in the art from the structural features of the relevant polynucleotide and/or context in which it is used.

"Readout" means a parameter, or parameters, which are measured and/or detected that can be converted to a number or value. In some contexts, readout may refer to an actual numerical representation of such collected or recorded data. For example, a readout of fluorescent intensity signals from a microarray is the address and fluorescence intensity of a signal being generated at each hybridization site of the microarray; thus, such a readout may be registered or stored in various ways, for example, as an image of the microarray, as a table of numbers, or the like.

"Reflex site", "reflex sequence" and equivalents are used to indicate sequences in a polynucleotide that are employed

to move a domain intramolecularly from its initial location to a different location in the polynucleotide. The sequence of a reflex site can be added to a polynucleotide of interest (e.g., present in an adapter ligated to the polynucleotide), be based on a sequence naturally present within the polynucleotide of interest (e.g., a genomic sequence in the polynucleotide), or a combination of both. The reflex sequence is chosen so as to be distinct from other sequences in the polynucleotide (i.e., with little sequence homology to other sequences likely to be present in the polynucleotide, e.g., genomic or sub-genomic sequences to be processed). As such, a reflex sequence should be selected so as to not hybridize to any sequence except its complement under the conditions employed in the reflex processes herein described. As described later in this application, the complement to the reflex sequence is inserted on the same strand of the polynucleotide (e.g., the same strand of a double-stranded polynucleotide or on the same single stranded polynucleotide) in a particular location so as to facilitate an intramolecular binding event on such particular strand. Reflex sequences employed in the reflex process described herein can thus have a wide range of lengths and sequences. Reflex sequences may range from 5 to 200 nucleotide bases in length.

"Solid support", "support", and "solid phase support" are used interchangeably and refer to a material or group of materials having a rigid or semi-rigid surface or surfaces. In many embodiments, at least one surface of the solid support will be substantially flat, although in some embodiments it may be desirable to physically separate synthesis regions for different compounds with, for example, wells, raised regions, pins, etched trenches, or the like. According to other embodiments, the solid support(s) will take the form of beads, resins, gels, microspheres, or other geometric configurations. Microarrays usually comprise at least one planar solid phase support, such as a glass microscope slide.

"Specific" or "specificity" in reference to the binding of one molecule to another molecule, such as a labeled target sequence for a probe, means the recognition, contact, and formation of a stable complex between the two molecules, together with substantially less recognition, contact, or complex formation of that molecule with other molecules. In one aspect, "specific" in reference to the binding of a first molecule to a second molecule means that to the extent the first molecule recognizes and forms a complex with another molecule in a reaction or sample, it forms the largest number of the complexes with the second molecule. Preferably, this largest number is at least fifty percent. Generally, molecules involved in a specific binding event have areas on their surfaces or in cavities giving rise to specific recognition between the molecules binding to each other. Examples of specific binding include antibody-antigen interactions, enzyme-substrate interactions, formation of duplexes or triplexes among polynucleotides and/or oligonucleotides, biotin-avidin or biotin-streptavidin interactions, receptor-ligand interactions, and the like. As used herein, "contact" in reference to specificity or specific binding means two molecules are close enough that weak noncovalent chemical interactions, such as Van der Waal forces, hydrogen bonding, base-stacking interactions, ionic and hydrophobic interactions, and the like, dominate the interaction of the molecules.

As used herein, the term "T_m" is used in reference to the "melting temperature." The melting temperature is the temperature (e.g., as measured in ° C.) at which a population of double-stranded nucleic acid molecules becomes half dissociated into single strands. Several equations for calculating the T_m of nucleic acids are known in the art (see e.g., Anderson and Young, Quantitative Filter Hybridization, in Nucleic Acid Hybridization (1985). Other references (e.g., Allawi, H. T. & SantaLucia, J., Jr., Biochemistry 36, 10581-94 (1997)) include alternative methods of computation which take structural and environmental, as well as sequence characteristics into account for the calculation of T_m.

"Sample" means a quantity of material from a biological, environmental, medical, or patient source in which detection, measurement, or labeling of target nucleic acids is sought. On the one hand it is meant to include a specimen or culture (e.g., microbiological cultures). On the other hand, it is meant to include both biological and environmental samples. A sample may include a specimen of synthetic origin. Biological samples may be animal, including human, fluid, solid (e.g., stool) or tissue, as well as liquid and solid food and feed products and ingredients such as dairy items, vegetables, meat and meat by-products, and waste. Biological samples may include materials taken from a patient including, but not limited to cultures, blood, saliva, cerebral spinal fluid, pleural fluid, milk, lymph, sputum, semen, needle aspirates, and the like. Biological samples may be obtained from all of the various families of domestic animals, as well as feral or wild animals, including, but not limited to, such animals as ungulates, bear, fish, rodents, etc. Environmental samples include environmental material such as surface matter, soil, water and industrial samples, as well as samples obtained from food and dairy processing instruments, apparatus, equipment, utensils, disposable and non-disposable items. These examples are not to be construed as limiting the sample types applicable to the present invention.

The terms "upstream" and "downstream" in describing nucleic acid molecule orientation and/or polymerization are used herein as understood by one of skill in the art. As such, "downstream" generally means proceeding in the 5' to 3' direction, i.e., the direction in which a nucleotide polymerase normally extends a sequence, and "upstream" generally means the converse. For example, a first primer that hybridizes "upstream" of a second primer on the same target nucleic acid molecule is located on the 5' side of the second primer (and thus nucleic acid polymerization from the first primer proceeds towards the second primer).

It is further noted that the claims may be drafted to exclude any optional element. As such, this statement is intended to serve as antecedent basis for use of such exclusive terminology as "solely", "only" and the like in connection with the recitation of claim elements, or the use of a "negative" limitation.

DETAILED DESCRIPTION OF THE INVENTION

The invention is drawn to compositions and methods for intramolecular nucleic acid rearrangement that find use in various applications of genetic analysis, including sequencing, as well as general molecular biological manipulations of polynucleotide structures.

Before the present invention is described, it is to be understood that this invention is not limited to particular embodiments described, as such may, of course, vary. It is also to be understood that the terminology used herein is for the purpose of describing particular embodiments only, and is not intended to be limiting, since the scope of the present invention will be limited only by the appended claims.

Where a range of values is provided, it is understood that each intervening value, to the tenth of the unit of the lower limit unless the context clearly dictates otherwise, between the upper and lower limits of that range is also specifically

disclosed. Each smaller range between any stated value or intervening value in a stated range and any other stated or intervening value in that stated range is encompassed within the invention. The upper and lower limits of these smaller ranges may independently be included or excluded in the range, and each range where either, neither or both limits are included in the smaller ranges is also encompassed within the invention, subject to any specifically excluded limit in the stated range. Where the stated range includes one or both of the limits, ranges excluding either or both of those included limits are also included in the invention.

Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. Although any methods and materials similar or equivalent to those described herein can be used in the practice or testing of the present invention, some potential and preferred methods and materials are now described. All publications mentioned herein are incorporated herein by reference to disclose and describe the methods and/or materials in connection with which the publications are cited. It is understood that the present disclosure supersedes any disclosure of an incorporated publication to the extent there is a contradiction.

It must be noted that as used herein and in the appended claims, the singular forms "a", "an", and "the" include plural referents unless the context clearly dictates otherwise. Thus, for example, reference to "a nucleic acid" includes a plurality of such nucleic acids and reference to "the compound" includes reference to one or more compounds and equivalents thereof known to those skilled in the art, and so forth.

The practice of the present invention may employ, unless otherwise indicated, conventional techniques and descriptions of organic chemistry, polymer technology, molecular biology (including recombinant techniques), cell biology, biochemistry, and immunology, which are within the skill of the art. Such conventional techniques include polymer array synthesis, hybridization, ligation, and detection of hybridization using a label. Specific illustrations of suitable techniques can be had by reference to the example herein below. However, other equivalent conventional procedures can, of course, also be used. Such conventional techniques and descriptions can be found in standard laboratory manuals such as *Genome Analysis: A Laboratory Manual Series* (Vols. I-IV), *Using Antibodies: A Laboratory Manual, Cells: A Laboratory Manual, PCR Primer: A Laboratory Manual, and Molecular Cloning: A Laboratory Manual* (all from Cold Spring Harbor Laboratory Press), Stryer, L. (1995) *Biochemistry* (4th Ed.) Freeman, New York, Gait, "*Oligonucleotide Synthesis: A Practical Approach*" 1984, IRL Press, London, Nelson and Cox (2000), Lehninger, A., *Principles of Biochemistry* 3rd Ed., W. H. Freeman Pub., New York, N.Y. and Berg et al. (2002) *Biochemistry,* 5th Ed., W. H. Freeman Pub., New York, N.Y., all of which are herein incorporated in their entirety by reference for all purposes.

The publications discussed herein are provided solely for their disclosure prior to the filing date of the present application. Nothing herein is to be construed as an admission that the present invention is not entitled to antedate such publication by virtue of prior invention. Further, the dates of publication provided may be different from the actual publication dates which may need to be independently confirmed.

As summarized above, aspects of the present invention are drawn to the use of a 'reflex' sequence present in a polynucleotide (e.g., in an adapter structure of the polynucleotide, in a genomic region of the polynucleotide, or a combination of both) to move a domain of the polynucleotide intra-molecularly from a first location to a second location. The reflex process described herein finds use in any number of applications, e.g., placing functional elements of a polynucleotide (e.g., sequencing primer sites and/or MID tags) into proximity to a desired sub-region of interest.

Nucleic Acids

The reflex process (as described in detail below) can be employed for the manipulation and analysis of nucleic acid sequences of interest from virtually any nucleic acid source, including but not limited to genomic DNA, complementary DNA (cDNA), RNA (e.g., messenger RNA, ribosomal RNA, short interfering RNA, microRNA, etc.), plasmid DNA, mitochondrial DNA, synthetic DNA, etc. Furthermore, any organism, organic material or nucleic acid-containing substance can be used as a source of nucleic acids to be processed in accordance with the present invention including, but not limited to, plants, animals (e.g., reptiles, mammals, insects, worms, fish, etc.), tissue samples, bacteria, fungi (e.g., yeast), phage, viruses, cadaveric tissue, archaeological/ancient samples, etc. In certain embodiments, the nucleic acids in the nucleic acid sample are derived from a mammal, where in certain embodiments the mammal is a human.

In certain embodiments, the nucleic acid sequences are enriched prior to the reflex sequence process. By enriched is meant that the nucleic acid is subjected to a process that reduces the complexity of the nucleic acids, generally by increasing the relative concentration of particular nucleic acid species in the sample (e.g., having a specific locus of interest, including a specific nucleic acid sequence, lacking a locus or sequence, being within a specific size range, etc.). There are a wide variety of ways to enrich nucleic acids having a specific characteristic(s) or sequence, and as such any convenient method to accomplish this may be employed. The enrichment (or complexity reduction) can take place at any of a number of steps in the process, and will be determined by the desires of the user. For example, enrichment can take place in individual parental samples (e.g., untagged nucleic acids prior to adaptor ligation) or in multiplexed samples (e.g., nucleic acids tagged with primer sites, MID and/or reflex sequences and pooled; MID are described in further detail below).

In certain embodiments, nucleic acids in the nucleic acid sample are amplified prior to analysis. In certain of these embodiments, the amplification reaction also serves to enrich a starting nucleic acid sample for a sequence or locus of interest. For example, a starting nucleic acid sample can be subjected to a polymerase chain reaction (PCR) that amplifies one or more region of interest. In certain embodiments, the amplification reaction is an exponential amplification reaction, whereas in certain other embodiments, the amplification reaction is a linear amplification reaction. Any convenient method for performing amplification reactions on a starting nucleic acid sample can be used in practicing the subject invention. In certain embodiments, the nucleic acid polymerase employed in the amplification reaction is a polymerase that has proofreading capability (e.g., phi29 DNA Polymerase, *Thermococcus litoralis* DNA polymerase, *Pyrococcus furiosus* DNA polymerase, etc.).

In certain embodiments, the nucleic acid sample being analyzed is derived from a single source (e.g., a single organism, virus, tissue, cell, subject, etc.), whereas in other embodiments, the nucleic acid sample is a pool of nucleic acids extracted from a plurality of sources (e.g., a pool of nucleic acids from a plurality of organisms, tissues, cells, subjects, etc.), where by "plurality" is meant two or more. As

such, in certain embodiments, a nucleic acid sample can contain nucleic acids from 2 or more sources, 3 or more sources, 5 or more sources, 10 or more sources, 50 or more sources, 100 or more sources, 500 or more sources, 1000 or more sources, 5000 or more sources, up to and including about 10,000 or more sources.

In certain embodiments, nucleic acid fragments that are to be pooled with nucleic acid fragments derived from a plurality of sources (e.g., a plurality of organisms, tissues, cells, subjects, etc.), where by "plurality" is meant two or more. In such embodiments, the nucleic acids derived from each source includes a multiplex identifier (MID) such that the source from which the each tagged nucleic acid fragment was derived can be determined. In such embodiments, each nucleic acid sample source is correlated with a unique MID, where by unique MID is meant that each different MID employed can be differentiated from every other MID employed by virtue of at least one characteristic, e.g., the nucleic acid sequence of the MID. Any type of MID can be used, including but not limited to those described in co-pending U.S. patent application Ser. No. 11/656,746, filed on Jan. 22, 2007, and titled "Nucleic Acid Analysis Using Sequence Tokens", as well as U.S. Pat. No. 7,393,665, issued on Jul. 1, 2008, and titled "Methods and Compositions for Tagging and Identifying Polynucleotides", both of which are incorporated herein by reference in their entirety for their description of nucleic acid tags and their use in identifying polynucleotides. In certain embodiments, a set of MIDs employed to tag a plurality of samples need not have any particular common property (e.g., T_m, length, base composition, etc.), as the asymmetric tagging methods (and many tag readout methods, including but not limited to sequencing of the tag or measuring the length of the tag) can accommodate a wide variety of unique MID sets.

In certain embodiments, each individual polynucleotide (e.g., double-stranded or single-stranded, as appropriate to the methodological details employed) in a sample to be analyzed is tagged with a unique MID so that the fate of each polynucleotide can be tracked in subsequent processes (where, as noted above, unique MID is meant to indicate that each different MID employed can be differentiated from every other MID employed by virtue of at least one characteristic, e.g., the nucleic acid sequence of the MID). For example (and as described below), having each nucleic acid tagged with a unique MID allows analysis of the sequence of each individual nucleic acid using the reflex sequence methods described herein. This allows the linkage of sequence information for large nucleic acid fragments that cannot be sequenced in a single sequencing run.

Reflex Sequence Process

As summarized above, aspects of the present invention include methods and compositions for moving a domain in a polynucleotide from a first location to a second location in the polynucleotide. An exemplary embodiment is shown in FIG. **1**A.

FIG. **1**A shows a single stranded polynucleotide **100** comprising, in a 5' to 3' orientation, a first domain (**102**; the domain to be moved); a reflex sequence **104**; a nucleic acid sequence **106** having a site distal to the first domain (Site A), and a complement of the reflex sequence **108** (positioned at the 3' terminus of the polynucleotide). The steps of the reflex method described below will move the first domain into closer proximity to Site A. It is noted here that the prime designation in FIG. **1**A denotes a complementary sequence of a domain. For example, First Domain' is the complement of the First Domain.

In Step **1**, the reflex sequence and its complement in the polynucleotide are annealed intramolecularly to form polynucleotide structure **112**, with the polynucleotide folding back on itself and hybridizing to form a region of complementarity (i.e., double stranded reflex/reflex' region). In this configuration, the 3' end of the complement of the reflex sequence can serve as a nucleic acid synthesis priming site. Nucleic acid synthesis from this site is then performed in extension Step **2** producing a complement of the first domain at the 3' end of the nucleic acid extension (shown in polynucleotide **114**; extension is indicated with dotted arrow labeled "extend").

Denaturation of polynucleotide **114** (e.g., by heat) generates linear single stranded polynucleotide **116**. As shown in FIG. **1**, resultant polynucleotide **116** contains a complement of the first domain at a position proximal to Site A (i.e., separated by only the complement of the reflex sequence). This resultant polynucleotide may be used for any subsequent analysis or processing steps as desired by the user (e.g., sequencing, as a template for amplification (linear, PCR, etc.), sequence specific extraction, etc.).

In alternative embodiments, the first domain and reflex sequence are removed from the 5' end of the double-stranded region of polynucleotide **114** (shown in polynucleotide **118**; removal is shown in the dotted arrow labeled "remove"). Removal of this region may be accomplished by any convenient method, including, but not limited to, treatment (under appropriate incubation conditions) of polynucleotide structure **114** with T7 exonuclease or by treatment with Lambda exonuclease; the Lambda exonuclease can be employed so long as the 5' end of the polynucleotide is phosphorylated. If the region is removed enzymatically, resultant polynucleotide **118** is used in place of polynucleotide **116** in subsequent steps (e.g., copying to reverse polarity).

In certain embodiments, polynucleotide **116** or **118** is used as a template to produce a double stranded polynucleotide, for example by performing a nucleic acid synthesis reaction with a primer that primes in the complement of the first domain. This step is sometimes referred to as copying to reverse polarity of a single stranded polynucleotide, and in some instances, the double-stranded intermediate product of this copying is not shown (see, e.g., FIG. **3**). For example, copying to reverse the polarity of polynucleotide **116** results in single-stranded polynucleotide **120** having, in a 5' to 3' orientation, the first domain (**122**); the reflex sequence (**124**); the complement of polynucleotide **106** (oriented with the complement of Site A (Site A'; **126**) proximal to the reflex sequence); the complement of the reflex sequence (**128**); and the complement of the first domain (**130**).

In certain embodiments, the first domain in the polynucleotide comprises one or more elements that find use in one or more subsequent processing or analysis steps. Such sequences include, but are not limited to, restriction enzyme sites, PCR primer sites, linear amplification primer sites, reverse transcription primer sites, RNA polymerase promoter sites (such as for T7, T3 or SP6 RNA polymerase), MID tags, sequencing primer sites, etc. Any convenient element can be included in the first domain and, in certain embodiments, is determined by the desires of the user of the methods described herein.

As an exemplary embodiment, suppose we want to sequence a specific polynucleotide region from multiple genomes in a pooled sample where the polynucleotide region is too long to sequence in a single reaction. For example, sequencing a polynucleotide region that is 2 kilobases or more in length using Roche 454 (Branford, Conn.)

technology, in which the length of a single sequencing run is about 400 bases. In this scenario, we can design a set of left hand primers (A_n) and right hand primers (B_n) specific for the polynucleotide region that are positioned in such a way that we can obtain direct sequences of all parts of the insert, as shown in FIG. **1**B. Note that the polynucleotide shown in FIG. **1**B (**140**) has a domain (**142**) containing a primer site and an MID denoting from which original sample(s) the polynucleotide is derived. Site **142** thus represents an example of a First Domain site such identified as **122** in the FIG. **1**A. The polynucleotide also includes a reflex site (**144**), which can be part of the polynucleotide region itself (e.g., a genomic sequence), added in a ligated adapter domain along with the primer site and the MID (an artificial sequence), or a combination of both (a sequence spanning the adapter/polynucleotide junction).

It is noted here that polynucleotide **140** can be categorized as a precursor to polynucleotide **100** in FIG. **1**A, as it does not include a 3' reflex sequence complementary to the reflex site (domain **108** in FIG. **1**A). As detailed below, polynucleotide **140** can be converted to a polynucleotide having the structural configuration of polynucleotide **100**, a polynucleotide suitable as a substrate for the reflex process described herein (e.g., by primer extension using a B_n primer and reversal of polarity).

In an exemplary embodiment, each A_n-B_n primer pair defines a nucleic acid region that is approximately 400 bases in length or less. This size range is within the single-sequencing run read length of the current Roche 454 sequencing platform; a different size range for the defined nucleic acid region may be utilized for a different sequencing platform. Thus, each product from each reflex process can be sequenced in a single run. It is noted here that primer pairs as shown in FIG. **1**B can be used to define regions **1** to **5** shown in FIG. **3** (described in further detail below).

In certain embodiments, to obtain the first part of the sequence of the polynucleotide region (i.e., in the original structure, that part of the polynucleotide closest to the first domain), we only need a right hand primer (e.g., B_0) and we do not need to transfer the MID as it is within reach of this sequencing primer (i.e., the MID is within 400 bases of sequencing primer B_0). All other B_n primers have the reflex sequence added to their 5' ends ("R" element shown on B primers) so that they read 5' reflex-B_n. However, in certain embodiments, the B_0 primer does include the reflex sequence and is used in the reflex process (along with a corresponding A_0 primer) as detailed below.

As described above, we obtain a single stranded polynucleotide having, in the 5' to 3' orientation, a primer site (e.g., for Roche 454 sequencing), an MID, a reflex sequence and the polynucleotide to be sequenced. Numerous methods for obtaining single-stranded polynucleotides of interest have been described and are known in the art, including in U.S. Pat. No. 7,217,522, issued on May 15, 2007; U.S. patent application Ser. No. 11/377,462, filed on Mar. 16, 2006; and U.S. patent application Ser. No. 12/432,080, filed on Apr. 29, 2009; each of which is incorporated by reference herein in their entirety. For example, a single stranded product can be produced using linear amplification with a primer specific for the primer site of the template. In certain embodiments, the primer includes a binding moiety to facilitate isolation of the single stranded nucleic acid of interest, e.g., to immobilize the top strand on a binding partner of the binding moiety immobilized on a solid support. Removal of a hybridized, non-biotinylated strand by denaturation using heat or high pH (or any other convenient method) serves to isolate the biotinylated strand. Binding moieties and their corresponding binding partners are sometimes referred to herein as binding partner pairs. Any convenient binding partner pairs may be used, including but not limited to biotin/avidin (or streptavidin), antigen/antibody pairs, etc.

It is noted here that while the figures and description of the reflex process provided herein depict manipulations with regard to a single stranded polynucleotide, it is not necessarily required that the single stranded polynucleotide described or depicted in the figures be present in the sample in an isolated form (i.e., isolated from its complementary strand). In other words, double stranded polynucleotides may be used where only one strand is described/depicted, which will generally be determined by the user.

The implementation of a single strand isolation step using the methods described above or variations thereof (or any other convenient single strand isolation step) will generally be based on the desires of the user. One example of isolating single stranded polynucleotides is shown in FIG. **2**. In this Figure, a starting double stranded template (with 5' to 3' orientation shown as an arrow) is denatured and primed with a biotinylated synthesis primer specific for the primer site. After extension of the primer (i.e., nucleic acid synthesis), the sample is contacted with a solid support having streptavidin bound to it. The biotin moiety (i.e., the binding partner of streptavidin) on the extended strands will bind to the solid-phase streptavidin. Denaturation and washing is then performed to remove all non-biotinylated polynucleotide strands. If desired, the bound polynucleotide, which can be used in subsequent reflex process steps (e.g., as a template for B_n primer extension reactions), may be eluted from the streptavidin support. Alternatively, the bound polynucleotide may be employed in subsequent steps of the desired process while still bound to the solid support (e.g., in solid phase extension reactions using B_n primers). This process, with minor variations depending on the template being used and the identity of the desired single stranded polynucleotide, may be employed at any of a number of steps in which a single stranded product is to be isolated. It is noted that in certain embodiments, substrate bound biotinylated polynucleotide can be used to produce and isolate non-biotinylated single stranded products (i.e., by eluting the non-biotinylated products while leaving the biotinylated templates bound to the streptavidin on the solid support). Thus, the specifics of how binding partners are used to isolate single stranded polynucleotides of interest will vary depending on experimental design parameters.

Additional single-stranded isolation/production methods include asymmetric PCR, strand-specific enzymatic degradation, and the use of in-vitro transcription followed by reverse transcriptase (IVT-RT) with subsequent destruction of the RNA strand. As noted above, any convenient single stranded production/isolation method may be employed.

To the single stranded polynucleotide shown in FIG. **1**B we anneal one of the B_n primers having the appended reflex sequence, denoted with a capital "R" (e.g., B_1) and extend the primer under nucleic acid synthesis conditions to produce a copy of the polynucleotide that has a reflex sequence at its 5' end. A single stranded copy of this polynucleotide is then produced to reverse polarity using a primer specific for the primer site in the first domain' (complement of the first domain **102**). The resulting nucleic acid has structure **100** shown in FIG. **1**A, where the first domain **102** includes the primer site and the MID. Site A (**110**) in FIG. **1** is determined by the specificity of the 5' reflex-B_n primer used.

The reflex process (e.g., as shown in FIG. **1**) is then performed to produce a product in which the primer site and

the MID are now in close proximity to the desired site (or region of interest (ROI)) within the original polynucleotide (i.e., the site defined by the primer used, e.g., B_1). The resulting polynucleotide can be used in subsequent analyses as desired by the user (e.g., Roche 454 sequencing technology).

It is noted here that, while not shown in FIGS. **1**A and **1**B, any convenient method for adding adapters to a polynucleotide to be processed as described herein may be used in the practice of the reflex process (adapters containing, e.g., primer sites, polymerase sites, MIDs, restriction enzyme sites, and reflex sequences). For example, adapters can be added at a particular position by ligation. For double stranded polynucleotides, an adapter can be configured to be ligated to a particular restriction enzyme cut site. Where a single stranded polynucleotide is employed, a double stranded adapter construct that possesses an overhang configured to bind to the end of the single-stranded polynucleotide can be used. For example, in the latter case, the end of a single stranded polynucleotide can be modified to include specific nucleotide bases that are complementary to the overhang in the double stranded adaptor using terminal transferase and specific nucleotides. In other embodiments, PCR or linear amplification methods using adapter-conjugated primers is employed to add an adapter at a site of interest. Again, any convenient method for producing a starting polynucleotide may be employed in practicing the methods of the subject invention.

In certain embodiments, the nucleic acid may be sequenced directly using a sequencing primer specific for the primer site. This sequencing reaction will read through the MID and desired site in the insert.

In certain embodiments, the polynucleotide may be isolated (or fractionated) using an appropriate A_n primer (e.g., when using B_1 as the first primer, primer A_1 can be used). In certain embodiments, the A_n primed polynucleotide is subjected to nucleic acid synthesis conditions to produce a copy of the fragment produced in the reflex process. In certain of these embodiments, the A_n primer has appended on its 5' end a primer site that can be used in subsequent steps, including sequencing reactions. Providing a primer site in the A_n primer allows amplifying and/or sequencing from both ends of the resultant fragment: from the primer site in the first domain **102** and the primer site in the A_n primer (not shown in FIG. **1**B). Because of the position of the primer sites and their distance apart (i.e., less than one sequencing run apart), sequencing from both ends will usually capture the sequence of the desired site (or ROI) and the sequence of the MID, which can be used for subsequent bioinformatic analyses, e.g., to positively identify the sample of origin. It is noted here that while sequencing in both directions is possible, it is not necessary, as sequencing from either primer site alone will capture the sequence of the ROI as well as its corresponding MID sequence.

Note that in certain embodiments, the first fragment obtained by amplification/extension from primer B_0 directly, the polarity of the ROI in the resulting fragment is reversed as compared to the ROI in fragments obtained by primers B_1-B_n. This is because the B_0-generated fragment, unlike the B_1-B_n generated fragments, has not been subjected to a reflex process which reverses the orientation of the ROI sequence with respect to the first domain/reflex sequence (as described above). Therefore, the B_0 primer may have appended to it a primer site (e.g., at its 5' end) that can be used for subsequent amplification and/or sequencing reactions (e.g., in Roche 454 sequencing system) rather than a reflex sequence as with primers B_1-B_n. However, in certain embodiments, as noted above, the reflex process may be used with a corresponding B_0-A_0 primer pair as described above, i.e., using a B_0 primer having a 5' reflex sequence and a corresponding A_0 primer with its corresponding 5' adapter domain (e.g., a primer site).

It is noted here that because the particular sections of sequence to be analyzed are defined by the A_n-B_n primer pairs (as shown and described above), a much higher sequence specificity is achieved as compared to using previous extraction methods that employ only a single oligo binding event (e.g., using probes on a microarray).

FIG. **3** provides a detailed flow chart for an exemplary embodiment that employs reflex sequences for use in sequencing multiple specific regions in a polynucleotide (i.e., regions **1**, **2**, **3**, **4** and **5** in an 11 kb region of lambda DNA).

A single parent DNA fragment **202** is generated that includes adapter domains (i.e., a Roche 454 sequencing primer site, a single MID, and a reflex sequence) and the sequence of interest. In the example shown, the sequence of interest is from lambda DNA and the reflex sequence is present on the top strand (with its complement shown in the bottom strand). Any convenient method for producing this parent DNA fragment may be used, including amplification with a primer that includes the adapter domains (e.g., using PCR), cloning the fragment into a vector that includes the adapter domains (e.g., a vector with the adapter domains adjacent to a cloning site), or by attaching adapters to polynucleotide fragments (e.g., fragment made by random fragmentation, by sequence-specific restriction enzyme digestion, or combinations thereof). While only a single fragment with a single MID is shown, the steps in FIG. **3** are applicable to samples having multiple different fragments each with a different MID, e.g., a sample having a population of homologous fragments from any number of different sources (e.g., different individuals). FIG. **3** describes the subsequent enzymatic steps involved in creating the five daughter fragments in which regions **1**, **2**, **3**, **4** and **5** (shown in polynucleotide **204**) are rearranged to be placed within a functional distance of the adapter domains (i.e., close enough to the adapter domains to be sequenced in a single Roche 454 sequencing reaction). Note that certain steps are shown for region **4** only (**206**).

In step **1**, the five regions of interest are defined within the parent fragment (labeled **1** to **5** in polynucleotide **204**) and corresponding primer pairs are designed for each. The distance of each region of interest from the reflex sequence is shown below polynucleotide **204**. The primer pairs are designed as described and shown in FIG. **1**B (i.e., the A_n-B_n primer pairs). For clarity, only primer sites for region **4** are shown in FIG. **3** ("primer sites" surrounding region **4**). In step **2**, sequence specific primer extensions are performed (only region **4** is shown) with corresponding B_n primers to produce single stranded polynucleotides having structure **208** (i.e., having the reflex sequence on the 5' terminus). As shown, the B_n primer for region **4** will include a sequence specific primer site that primes at the 3'-most primer site noted for region **4** (where "3'-most" refers to the template strand, which in FIG. **3** is the top strand). This polynucleotide is copied back to produce polynucleotide **210** having reversed polarity (e.g., copied using a primer that hybridizes to the 454A' domain). Polynucleotide **210** has structure similar to polynucleotide **100** shown at the top of FIG. **1**. Step **4** depicts the result of the intramolecular priming between the reflex sequence and its complement followed by extension to produce the MID' and 454A' structures at the 3' end (polynucleotide **212**). In the embodiments shown in

FIG. **3**, polynucleotide **212** is treated with T7 exonuclease to remove double stranded DNA from the 5' end (as indicated above, this step is optional). The polynucleotide formed for region **4** is shown as **216** with polynucleotides for the other regions also shown (**214**).

It is noted here that the formation of each of the polynucleotides **214** may be accomplished either in separate reactions (i.e., structure with region **1** in proximity to the adapter domains is in a first sample, the structure with region **2** in proximity to the adapter region is in a second sample, etc.) or in one or more combined sample.

In step **6** the polynucleotides **214** are copied to reverse polarity to form polynucleotides **218**. In step **7**, each of these products are then primed with the second primer of the specific primer pair (see A_n primers as shown in FIG. **1**B) each having a second Roche 454 primer site (454B) attached at the 5' end, and extended to form products **220**. Steps **6** and **7** may be combined (e.g., in a single PCR or other amplification reaction).

In summary, FIG. **3** shows how the reflex process can be employed to produce five daughter fragments **220** of similar length (e.g., ~500 bp) each of which contain DNA sequences that differ in their distance from the reflex sequence in the starting structure **202** while maintaining the original MID.

FIG. **4** shows another exemplary use of the reflex process as described herein. In the embodiment shown in FIG. **4**, a target sequence (i.e., containing region of interest "E") is enriched from a pool of adapter-attached fragments. In certain embodiments, the fragments are randomly sheared, selected for a certain size range (e.g., DNA having a length from 100 to 5000 base pairs), and tagged with adapters (e.g., asymmetric adapters, e.g., as described in U.S. patent application Ser. No. 12/432,080, filed on Apr. 29, 2009). The asymmetric adaptor employed in FIG. **4** contains a sequencing primer site (454A, as used in the Roche 454 sequencing platform), an MID, an X sequence, and an internal stem region (ISR), which denotes the region of complementarity for the asymmetric adapter that is adjacent to the adapter attachment site (see, e.g., the description in U.S. application Ser. No. 12/432,080, filed on Apr. 29, 2009, incorporated herein by reference in its entirety). The X sequence can be any sequence that can serve as a binding site for a polynucleotide containing the complement of the X sequence (similar to a primer site). As described below, the X sequence allows for the annealing of an oligonucleotide having a 5' overhang that can serve as a template for extension of the 3' end of the adaptor oligonucleotide. The sequencing direction of the sequencing primer site (454A primer site in structure **401** of FIG. **4**) is oriented such that amplification of the adapter ligated fragment using the sequencing primer site proceeds away from the ligated genomic insert. This has the effect of making the initial asymmetric adapter ligated library 'inert' to amplification using this primer, e.g., in a PCR reaction.

To extract a region of interest (the "E" region), the library is mixed with an oligonucleotide (**403**) containing a 3' X' sequence and a target specific priming sequence (the 1' sequence) under hybridization/annealing conditions. The target specific sequence 1' is designed to flank one side of the region of interest (the 1' sequence adjacent to E in the genomic insert; note that only the E-containing polynucleotide fragment is shown in FIG. **4**), much like a PCR primer. After annealing primer **403**, the hybridized complex is extended, whereby all of the adaptor tagged fragments will obtain the complement of the target specific sequence (i.e., the 1 sequence) on the 3' end (see structure **405**; arrows denote the direction of extension).

Extended products **405** are then denatured and the 1/1' regions allowed to hybridize intramolecularly in a reflex process priming event, after which nucleic acid extension is performed to form structure **407** (extension is from the 1 priming site; shown with an arrow). This reflex reaction creates a product (**407**) that, unlike its parent structure (**405**), has a sequencing primer site (454A) that is oriented such the extension using this primer sequence proceeds towards the region of interest. Thus, in the absence of a priming and extension reflex reaction, extension with a sequencing primer will not generate a product containing the region of interest (the E region). In other words, only E-region containing target polynucleotides will have a 454A sequence that can amplify genomic material (structure **407**).

After completing the reflex process (using 1/1' as the reflex sequences), a PCR amplification reaction is performed to amplify the region of interest (with associated adapter domains). However, before performing the PCR reaction, the fragment sample is "inactivated" from further extension using terminal transferase and ddNTPs. This inactivation prevents non-target adaptor tagged molecules from performing primer extension from the 3' primer **1** site. Once inactivated, a PCR reaction is performed using a sequencing primer (i.e., 454A primer **409**) and a second primer that primes and extends from the opposite side of the region of interest (i.e., primer **411**, which includes a 5' 454B sequencing primer site and a 3' "2" region that primes on the opposite end of E from the 1 region). Only fragments that have undergone the reflex process and contain the E region will be suitable templates for the PCR reaction and produce the desired product (**413**).

Thus, the process exemplified in FIG. **4** allows for the movement of an adapter domain (e.g., containing functional elements and/or MID) into proximity to a desired region of interest.

The reflex process described herein can be used to perform powerful linkage analysis by combining it with nucleic acid counting methods. Any convenient method for tagging and/or counting individual nucleic acid molecules with unique tags may be employed (see, e.g., U.S. Pat. No. 7,537,897, issued on May 26, 2009; U.S. Pat. No. 7,217,522, issued on May 15, 2007; U.S. patent application Ser. No. 11/377,462, filed on Mar. 16, 2006; and U.S. patent application Ser. No. 12/432,080, filed on Apr. 29, 2009; each of which is incorporated by reference herein in their). All of this can be conducted in parallel thus saving on the cost of labor, time and materials.

In one exemplary embodiment, a large collection of sequences is tagged with MID such that each polynucleotide molecule in the sample has a unique MID. In other words, each polynucleotide in the sample (e.g., each individual double stranded or single stranded polynucleotide) is tagged with a MID that is different from every other MID on every other polynucleotide in the sample. In general, to accomplish such molecular tagging the number of distinct MID tags to be used should be many times greater than the actual number of molecules to be analyzed. This will result in the majority of individual nucleic acid molecules being labeled with a unique ID tag (see, e.g., Brenner et al., Proc. Natl. Acad. Sci. USA. 2000 97(4):1665-70). Any sequences that then result from the reflex process on that particular molecule (e.g., as described above) will thus be labeled with the same unique MID tag and thus inherently linked. Note that once all molecules in a sample are individually tagged, they can be manipulated and amplified as much as needed for processing so long as the MID tag is maintained in the products generated.

For example, we might want to sequence one thousand viral genomes (or a specific genomic region) or one thousand copies of a gene present in somatic cells. After tagging each polynucleotide in the sample with a sequencing primer site, MID and reflex sequence (as shown in the figures and described above), we use the reflex process to break each polynucleotide into lengths appropriate to the sequencing procedure being used, transferring the sequencing primer site and MID to each fragment (as described above). Obtaining sequence information from all of the reflex-processed samples can be used to determine the sequence of each individual polynucleotide in the starting sample, using the MID sequence to defining linkage relationships between sequences from different regions in the polynucleotide being sequenced. Using a sequencing platform with longer read lengths can minimize the number of primers to be used (and reflex fragments generated).

The advantages noted above are shown in FIG. **5**. This figure shows a comparison of methods for identifying nucleic acid polymorphisms in homologous nucleic acids in a sample (e.g., the same region derived from a chromosomal pair of a diploid cell or viral genomes/transcripts). The top schematic shows two nucleic acid molecules in a sample (**1** and **2**) having a different assortment of polymorphisms in polymorphic sites A, B and C (A**1**, B**1**, C**1** and C**2**). Standard sequencing methods using fragmentation (left side) can identify the polymorphisms in these nucleic acids but do not retain linkage information. Employing the reflex process described herein to identify polymorphisms (right side) maintains linkage information. It is noted that not all domain structures and steps are shown in the reflex process for simplicity.

Kits and Systems

Also provided by the subject invention are kits and systems for practicing the subject methods, as described above, such vectors configured to add reflex sequences to nucleic acid inserts of interest and regents for performing any steps in the cloning or reflex process described herein (e.g., restriction enzymes, nucleotides, polymerases, primers, exonucleases, etc.). The various components of the kits may be present in separate containers or certain compatible components may be precombined into a single container, as desired.

The subject systems and kits may also include one or more other reagents for preparing or processing a nucleic acid sample according to the subject methods. The reagents may include one or more matrices, solvents, sample preparation reagents, buffers, desalting reagents, enzymatic reagents, denaturing reagents, where calibration standards such as positive and negative controls may be provided as well. As such, the kits may include one or more containers such as vials or bottles, with each container containing a separate component for carrying out a sample processing or preparing step and/or for carrying out one or more steps of a nucleic acid variant isolation assay according to the present invention.

In addition to above-mentioned components, the subject kits typically further include instructions for using the components of the kit to practice the subject methods, e.g., to prepare nucleic acid samples for perform the reflex process according to aspects of the subject methods. The instructions for practicing the subject methods are generally recorded on a suitable recording medium. For example, the instructions may be printed on a substrate, such as paper or plastic, etc. As such, the instructions may be present in the kits as a package insert, in the labeling of the container of the kit or components thereof (i.e., associated with the packaging or sub-packaging) etc. In other embodiments, the instructions are present as an electronic storage data file present on a suitable computer readable storage medium, e.g. CD-ROM, diskette, etc. In yet other embodiments, the actual instructions are not present in the kit, but means for obtaining the instructions from a remote source, e.g. via the internet, are provided. An example of this embodiment is a kit that includes a web address where the instructions can be viewed and/or from which the instructions can be downloaded. As with the instructions, this means for obtaining the instructions is recorded on a suitable substrate.

In addition to the subject database, programming and instructions, the kits may also include one or more control samples and reagents, e.g., two or more control samples for use in testing the kit.

Utility

The reflex process described herein provides significant advantages in numerous applications, a few of which are noted below (as well as described above).

For example, as described above, certain aspects of the reflex process define the particular sections of sequence to be analyzed by a primer pair, as in PCR (e.g., the two oligos shown as A_n-B_n in FIG. **1**B). This results in higher sequence specificity as compared to other extraction methods (e.g., using probes on a microarray) that only use a single oligo sequence. The separation of the probes defines a length that can be relatively uniform (hence making subsequent handling including amplification more uniform) and can also be tailored to the particular sequencing platform being employed.

Further, as described above, aspects of the present invention can be used to analyze homologous genomic locations in a multiplexed sample (i.e., a sample having polynucleotides from different genomic samples) in which the polynucleotides are tagged with the MID. This is possible because the reflex process, which operates intramolecularly, maintains the MID thus linking any particular fragment to the sample from which it originates.

Finally, as the reflex processes described herein function intramolecularly, one can determine the genetic linkage between different regions on the same large fragment that are too far apart to be sequenced in one sequence read. Such a determination of linkage may be of great value in plant or animal genetics (e.g., to decide if a particular set of variations are linked together on the same stretch of chromosome) or in viral studies (e.g., to determine if particular variations are linked together on the same stretch of a viral genome/transcripts, e.g., HIV, hepatitis virus, etc.).

EXAMPLES

Example I

FIGS. **6** and **7** provide experimental data and validation of the reflex process described herein using synthetic polynucleotide substrates.

Methods

Substrate:

The 100 base oligonucleotide substrate (as shown diagrammatically in FIG. **6**A) was synthesized with internal fluorescein-dT positioned between the REFLEX and REFLEX' sequences. This label provides convenient and sensitive method of detection of oligonucleotide species using polyacrylamide gel electrophoresis.

Extension Reactions:

Reactions were prepared which contained 1 μM of the 100 base oligonucleotide substrate, 200 μM dNTPs, presence or

absence of 1 μM competitor oligonucleotide, 0.5 μl of each DNA polymerase ("DNAP"): Vent (NEB, 2 units/μl), Taq (Qiagen HotStarTaq 5 units/μl) and Herculase (Stratagene), and made up to 50 μl with the appropriate commercial buffers for each polymerase and dH_2O. For Taq titrations 0.5 μl, 1 μl, 2 μl, and 3 μl enzyme was used in the same 50 μl volume. Reactions were heated in a Biometra thermocycler to 95° C. for 15 minutes (Taq) or 5 minutes (Herculase, Vent), followed by 55° C. or 50° C. for 30 seconds, and a final incubation at 72° C. for 10 minutes.

T7 Exonuclease Digestions:

Reactions were prepared with 10 μl extension reactions above, 0.5 μl T7 exonuclease (NEB, 10 units/μl), and made up to 50 μl using NEB Buffer 4 and dH_2O. Reactions were incubated at 25° C. for 30 minutes.

Gel Electrophoresis Analysis:

An 8% denaturing polyacrylamide gel was used to analyze reaction species. 0.4 μl of extension reactions, and 2 μl of digestion reactions were loaded and ran at 800V for ~1.5 hours. Gels were analyzed for fluorescein using an Amersham/General Electric Typhoon imager.

Results

FIG. **6**A shows the structure of each stage of reflex sequence processing with the expected nucleic acid size shown on the left. The initial single stranded nucleic acid having a sequencing primer site (the Roche 454 sequencing primer A site; listed as 454A); an MID; a reflex sequence; the insert; and a complement of the reflex sequence is 100 nucleotides in length. After self-annealing and extension, the product is expected to be 130 nucleotides in length. After removal of the double stranded region from the 5' end, the nucleic acid is expected to be 82 bases in length.

FIG. **6**B shows the results of three experiments using three different nucleic acid polymerases (Vent, Herculase and Taq, indicated at the top of the lanes). The temperature at which the annealing was carried out is shown at the top of each lane (either 50° C. or 55° C.). The sizes of the three nucleic acids as noted above are indicated on the left and right side of the gel.

As shown in FIG. **6**B, extension appears to be most efficient under the conditions used with Herculase (Herculase is a mixture of two enzymes: modified Pfu DNAP and Archaemax (dUTPase)). Most (or all) of the initial 100 base pair nucleic acid are converted to the 130 base pair product (see lanes 6 and 7). However, after T7 exonuclease digestion the 3'-5' exonuclease activity of Herculase results in partial digestion of the desired 82 base product (note bands at and below the 82 base pairs in lanes 8 and 9).

Taq, which lacks 3'-5' exonuclease activity, shows a stronger band at the expected size of the final product after T7 exonuclease digestion (see lane 13).

FIG. **7** shows the effect on the reflex process of increasing amounts of Taq polymerase as well as the use of a reflex sequence competitor (schematically shown in FIG. **7**A).

As shown in lanes 2 to 5, increased Taq concentration improves extension to ~90% conversion of the starting nucleic acid (see lane 5). Lanes 7 to 8 show that T7 exonuclease digestion does not leave a perfect 82 base product. This may be due to collapse of dsDNA when T7 exonuclease has nearly completed its digestion from the 5' end in the double stranded region of the fold-back structure. It is noted that in many embodiments, the removal of a few additional bases from the 5' end of the polynucleotide will not interfere with subsequent analyses, as nucleotide bases at the 5' end are often removed during subsequent steps.

As shown in Lanes 11-14, addition of a competitor (which can interfere with annealing of the reflex sequences to form the fold-back structure) results in only a small decrease (~5-10%) of fully extended product. Thus, as expected, the intramolecular reaction is heavily favored. Although not shown, we have observed that the competitor oligonucleotide also gets extended by the same amount (~5-10%).

The concentration of the competitor, the concentration of the reflex substrate, and the overall genetic complexity, will all likely affect specific results. The experiments shown in FIGS. **6** and **7** demonstrate that the core parts of the reflex processes as described herein is functional and can be implemented.

Example II

FIG. **8** shows the reflex workflow (diagram at left) and exemplary results of the workflow (gel at right) for a specific region of interest (ROI). The starting material is a double stranded nucleic acid molecule (**700**) that contains a 454A primer site, an MID, a reflex site, and a polynucleotide of interest having three ROIs (**2**, **3** and **4**) at different locations therein. This starting material was subjected to reflex processes (as described in above) specific for ROI **2** as shown in the diagram at the left of the figure, both with and without the use of a T7 exonuclease step (the T7 exonuclease step is shown in the diagram is indicated as "Optional").

Completion of all steps shown in the reflex process should result in a double stranded polynucleotide of 488 base pairs (**702**) with or without the T7 exonuclease step.

As shown in the gel on the right of FIG. **8**, the 488 base pair product was produced in reflex processes with and without the T7 exonuclease step.

FIG. **9** shows an exemplary protocol for a reflex process based on the results discussed above. The diagram shows specific reflex process steps with indications on the right as to where purification of reaction products is employed (e.g., using Agencourt SPRI beads to remove primer oligos). One reason for performing such purification steps is to reduce the potential for generating side products in a reaction (e.g., undesirable amplicons). While FIG. **9** indicates three purification steps, fewer or additional purification steps may be employed depending on the desires of the user. It is noted that the steps of reversing polarity, reflex priming and extension, and "stretch out" (or denaturation)/second reversing polarity step can be performed without intervening purification steps.

The protocol shown in FIG. **9** includes the following steps:

annealing a first primer containing a 5' reflex sequence (or reflex tail, as noted in the figure) specific for the 3' primer site for the R' region to the starting polynucleotide and extending (the primer anneals to the top strand at the primer site at the right of R in polynucleotide **902**, indicated with a *; this step represents the first denature, anneal and extend process indicated on the right);

after purification, adding a 454A primer and performing three cycles of denaturing, annealing and extending: the first cycle results in the copy-back from the 454A primer to reverse the polarity of the strand just synthesized; the second cycle breaks apart the double stranded structure produced, allows the reflex structure to form and then extend; the third cycle results in another copy-back using the same 454A primer originally added;

after purification, adding a second primer specific for the second primer site for the R' region having a 5' 454B tail (this primer anneals to the primer site 3' of the R' region in polynucleotide **904**, indicated with a *) and denaturing, annealing and extending resulting in a polynucleotide prod-

uct having 454A and 454B sites surrounding the MID, the reflex sequence, and R'. Note that the first primer specific for the R' region and the second primer specific for the R' region define its boundaries, as described above and depicted in FIG. **1**B);

after another purification, adding 454A and 454B primers and performing a PCR amplification reaction.

Example III

As described above, a reflex sequence can be an "artificial" sequence added to a polynucleotide as part of an adapter or can be based on a sequence present in the polynucleotide of interest being analyzed, e.g., a genomic sequence (or "non-artificial").

The data shown in prior Examples used "artificial" reflex sites. In this Example, the reflex site is a genomic sequence present in the polynucleotide being analyzed.

The starting material is a double stranded DNA containing a 454A site, an MID and a polynucleotide to be analyzed. The 454A and MID were added by adapter ligation to parent polynucleotide fragments followed by enrichment of the polynucleotide to be analyzed by a hybridization-based pull-out reaction and subsequent secondary PCR amplification (see Route **1** in FIG. **13**). Thus, the reflex site employed in this example is a sequence normally present at the 5' end of the subject polynucleotide (a genomic sequence). The polynucleotide being analyzed includes a region of interest distal to the 454A and MID sequences that is 354 base pairs in length.

This starting double stranded nucleic acid is 755 base pairs in length. Based on the length of each of the relevant domains in this starting nucleic acid, the reflex process should result in a product of 461 base pairs.

FIG. **10** shows the starting material for the reflex process (left panel) and the resultant product generated using the reflex process (right panel; reflex process was performed as described in Example II, without using a T7 exonuclease step). A size ladder is included in the left hand lane of each gel to allow estimation of the size of the test material. This figure shows that the 755 base pair starting nucleic acid was processed to the expected 461 base pair product, thus confirming that a "non-artificial" reflex site is effective in moving an adapter domain from one location to another in a polynucleotide of interest in a sequence specific manner.

Example IV

FIG. **11** shows a schematic of an experiment in which the reflex process is performed on a single large initial template (a "parent" fragment) to generate 5 different products ("daughter" products) each having a different region of interest (i.e., daughter products are produced having either region **1**, **2**, **3**, **4** or **5**). The schematic in FIG. **11** shows the starting fragment (11,060 base pairs) and resulting products (each 488 base pairs) generated from each of the different region of interest-specific reflex reactions (reflex reactions are performed as described above). The panel (gel) on the bottom of FIG. **11** shows the larger starting fragment (Lane 1) and the resulting daughter products for each region-specific reflex reaction (lanes 2 to 6, with the region of interest noted in each in the box), where the starting and daughter fragments have the expected lengths. Sequencing of the products confirmed the identity of the region of interest in each of the reflex products shown in the gel. These results demonstrate that multiple different reflex products can be generated from a single, asymmetrically tagged parent fragment while maintaining the adapter domains (e.g., the primer sites and MID).

Example V

FIG. **12** details experiments performed to determine the prevalence of intramolecular rearrangement (as desired in the reflex process) vs. intermolecular rearrangement. Intermolecular rearrangement is undesirable because it can lead to the transfer of an MID from one fragment to another (also called MID switching). MID switching can occur if a reflex sequence in a first fragment hybridizes to its complement in a second fragment during the reflex process, leading to appending the MID from the second fragment to the first fragment. Thus, intermolecular rearrangement, or MID switching, should be minimized to prevent the transfer of an MID from one fragment in the sample to another, which could lead to a misrepresentation of the source of a fragment.

To measure the prevalence of MID switching under different reflex conditions, fragments having different sizes were generated that included two different MIDs, as shown in the top panel of FIG. **12**. The common sequence on these fragments serves as the priming site for the first extension reaction to add the second reflex sequence (see, e.g., step **2** of FIG. **3**). Three exemplary fragments are shown in FIG. **12** for each different fragment size (i.e., 800 base pairs with an MIDB and MIDA combination; 1900 base pairs with MDC and MIDA combination; and 3000 base pairs with MIDD and MIDA combination). For each MID family (A, B, C and D), there are 10 different members (i.e., MIDA had 10 different members, MIDB has 10 different members, etc.). A set of 10 dual MID fragments for each different size fragment (i.e., 800, 1900 and 3000 base pairs) were generated, where the MID pairs (i.e., MIDA/MIDB, MIDA/MIDC, and MIDA/MIDD) were designated as 1/1, 2/2, 3/3, 4/4, 5/5, 6/6, 7/7, 8/8, 9/9, and 10/10. All 10 fragments of the same size were then mixed together and a reflex protocol was performed.

Due to the domain structure of the fragments, a successful reflex process results in the two MIDs for each fragment being moved to within close enough proximity to be sequenced in a single read using the Roche 454 sequencing platform (see the reflex products shown in the schematic of FIG. **12**). The reflex reactions for each fragment size were performed at four different fragment concentrations to determine the effect of this parameter, as well as fragment length, in the prevalence of MID switching. The reflex products from each reaction performed were subjected to 454 sequencing to determine the identity of both MIDs on each fragment, and thereby the proportion of MID switching that occurred.

The panel on the bottom left of FIG. **12** shows the rate of MID switching (Y axis, shown in % incorrect (or switched) MID pair) for each different length fragment at each different concentration (X axis; 300, 30, 3 and 0.3 nM). As shown in this panel, the MID switch rate decreases with lower concentrations, as would be expected, because intermolecular, as opposed to intramolecular, binding events are concentration dependent (i.e., lower concentrations lead to reduced intermolecular hybridization/binding). In addition, the MID switch rate decreases slightly with length. This is somewhat unexpected as the ends of longer DNA fragments are effectively at a lower concentration with respect to one another. The reasons for why we do not see this is probably because the production of reflex priming intermediates continues during the final PCR, which means that reflex priming

reactions are happening continuously which contributes to MID switching. It is probably the case that the shorter reflex products are able to undergo a higher rate of 'background' reflexing, and therefore increase the overall MID switch rate a little.

These results demonstrate that MID switching can be minimized (e.g., to below 2%, below 1% or even to nearly undetectable levels) by altering certain parameters of the reaction, e.g., by reducing fragment concentration and/or fragment length.

The panel on the bottom right of FIG. **12** shows the frequency of MID switching in the reflex process for the 800 base pair fragments (i.e., MIDA/MIDB containing fragments). In this figure, the area of each circle is proportional to the number of reads containing the corresponding MIDA and MIDB species (e.g., MIDA**1**/MIDB**1**; MIDA**1**/MIDB**2**; etc.). Thus, a circle representing 200 reads will be 40 times larger in terms of area than a circle representing 5 reads.

As noted above, the MIDA/MIDB combinations having the same number (shown on the X and Y axis, respectively) represent the MIDA/MIDB combinations present in the sample prior to the reflex process being performed (i.e., MIDA/MIDB combinations 1/1, 2/2, 3/3, 4/4, 5/5, 6/6, 7/7, 8/8, 9/9, and 10/10 were present in the starting sample). All other MIDA/MIDB combinations identified by Roche 454 sequencing were the result of MID switching.

This figure shows that the MID switching that occurs during the reflex process is random, i.e., that MID switching is not skewed based on the identity of the MIDs in the reaction).

Exemplary Reflex Protocols

FIG. **13** shows a diagram of exemplary protocols for performing the reflex process on pools of nucleic acids, for example, pools of nucleic acids from different individuals, each of which are labeled with a unique MID. In Route **3**, a pooled and tagged extended library is subjected directly to a reflex process. In Route **2**, the pooled library is enriched by target-specific hybridization followed by performing the reflex process. In Route **1** employs enrichment by PCR amplification. As shown in FIG. **13**, PCR enrichment can be performed directly on the pooled tagged extended library or in a secondary PCR reaction after a hybridization-based enrichment step has been performed (as in Route **2**) to generate an amplicon substrate that is suitable for the reflex process. Additional routes for preparing a polynucleotide sample for performing a reflex process can be implemented (e.g., having additional amplification, purification, and/or enrichment steps), which will generally be dependent on the desires of the user.

Although the foregoing invention has been described in some detail by way of illustration and example for purposes of clarity of understanding, it is readily apparent to those of ordinary skill in the art in light of the teachings of this invention that certain changes and modifications may be made thereto without departing from the spirit or scope of the appended claims.

Accordingly, the preceding merely illustrates the principles of the invention. It will be appreciated that those skilled in the art will be able to devise various arrangements which, although not explicitly described or shown herein, embody the principles of the invention and are included within its spirit and scope. Furthermore, all examples and conditional language recited herein are principally intended to aid the reader in understanding the principles of the invention and the concepts contributed by the inventors to furthering the art, and are to be construed as being without limitation to such specifically recited examples and conditions. Moreover, all statements herein reciting principles, aspects, and embodiments of the invention as well as specific examples thereof, are intended to encompass both structural and functional equivalents thereof. Additionally, it is intended that such equivalents include both currently known equivalents and equivalents developed in the future, i.e., any elements developed that perform the same function, regardless of structure. The scope of the present invention, therefore, is not intended to be limited to the exemplary embodiments shown and described herein. Rather, the scope and spirit of present invention is embodied by the appended claims.

What is claimed:

1. A method of analyzing nucleic acids from a plurality of single cells, the method comprising:

(a) providing a sample comprising a plurality of single cells, wherein each single cell of the plurality of single cells comprises a plurality of sample polynucleotides;

(b) generating a plurality of tagged polynucleotides from the plurality of sample polynucleotides, wherein each tagged polynucleotide comprises:

(i) a sequence from a sample polynucleotide of the plurality of sample polynucleotides; and

(ii) a multiplex identifier (MID) sequence comprising:

I. a first tag sequence associated with the single cell from which the sample polynucleotide is derived, wherein the first tag sequence is a different sequence for different single cells in the plurality of single cells; and

II. a second tag sequence distinguishing the sample polynucleotide from other sample polynucleotides derived from the same single cell;

(c) sequencing the plurality of tagged polynucleotides to obtain a plurality of identified polynucleotide sequences;

(d) using the first tag sequence to correlate the identified polynucleotide sequence with the single cell from which the identified polynucleotide sequence is derived; and

(e) using the second tag sequence to correlate the identified polynucleotide sequence with the sample polynucleotide from which the identified polynucleotide sequence is derived.

2. The method of claim **1**, wherein the method further comprises amplifying the tagged polynucleotides prior to the sequencing step (c).

3. The method of claim **1**, wherein the sample polynucleotides are selected from DNA and RNA.

4. The method of claim **3**, wherein the sample polynucleotides comprise mRNA.

5. The method of claim **1**, wherein the tagged polynucleotides are generated through at least one ligation reaction.

6. The method of claim **1**, wherein the tagged polynucleotides are generated through PCR or linear amplification of the plurality of sample polynucleotides using an adapter sequence comprising the MID sequence and an amplification primer.

* * * * *

Exhibit B

US010240197B1

(12) **United States Patent**
Brenner et al.

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(45) **Date of Patent: *Mar. 26, 2019**

(54) **METHODS FOR ANALYZING NUCLEIC ACIDS FROM SINGLE CELLS**

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(*) Notice: Subject to any disclaimer, the term of this patent is extended or adjusted under 35 U.S.C. 154(b) by 0 days.

This patent is subject to a terminal disclaimer.

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Related U.S. Application Data

(63) Continuation of application No. 15/677,957, filed on Aug. 15, 2017, now Pat. No. 10,155,981, which is a continuation of application No. 14/792,094, filed on Jul. 6, 2015, now abandoned, which is a continuation of application No. 14/172,694, filed on Feb. 4, 2014, now Pat. No. 9,102,980, which is a continuation of application No. 14/021,790, filed on Sep. 9, 2013, now Pat. No. 8,679,756, which is a continuation of application No. 13/859,450, filed on Apr. 9, 2013, now Pat. No. 8,563,274, which is a continuation of application No. 13/622,872, filed on Sep. 19, 2012, now abandoned, which is a continuation of application No. 13/387,343, filed as application No. PCT/IB2010/002243 on Aug. 13, 2010, now Pat. No. 8,298,767.

(60) Provisional application No. 61/288,792, filed on Dec. 21, 2009, provisional application No. 61/235,595, filed on Aug. 20, 2009.

(51) **Int. Cl.**

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C12Q 1/686	(2018.01)
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C12N 15/10	(2006.01)
C12Q 1/6855	(2018.01)

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CPC ***C12Q 1/6874*** (2013.01); ***C12N 15/1065*** (2013.01); ***C12Q 1/686*** (2013.01); ***C12Q 1/6806*** (2013.01); ***C12Q 1/6855*** (2013.01)

(58) **Field of Classification Search**
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See application file for complete search history.

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(57) **ABSTRACT**

Aspects of the present invention include analyzing nucleic acids from single cells using methods that include using tagged polynucleotides containing multiplex identifier sequences.

26 Claims, 13 Drawing Sheets

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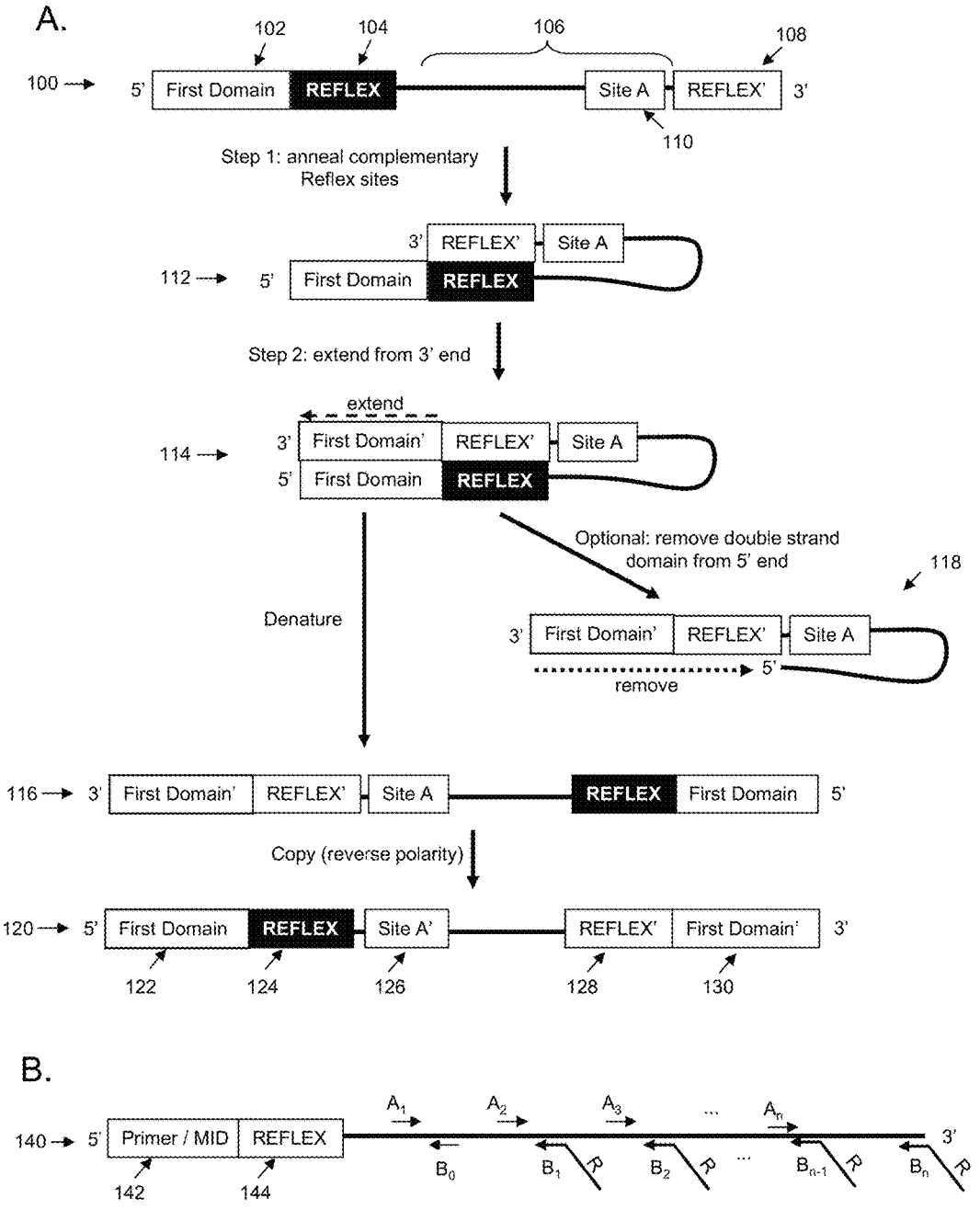

A.
102
104
106
108
100
5'
First Domain
REFLEX
Site A
REFLEX'
3'
110
Step 1: anneal complementary Reflex sites
3'
REFLEX'
Site A
112
5'
First Domain
REFLEX
Step 2: extend from 3' end
extend
114
3'
First Domain'
REFLEX'
Site A
5'
First Domain
REFLEX
Optional: remove double strand domain from 5' end
118
Denature
3'
First Domain'
REFLEX'
Site A
5'
remove
116
3'
First Domain'
REFLEX'
Site A
REFLEX
First Domain
5'
Copy (reverse polarity)
120
5'
First Domain
REFLEX
Site A'
REFLEX'
First Domain'
3'
122
124
126
128
130
B.
140
5'
Primer / MID
REFLEX
142
144
A1
A2
A3
An
3'
B0
B1
R
B2
R
Bn-1
R
Bn
R

Fig. 1

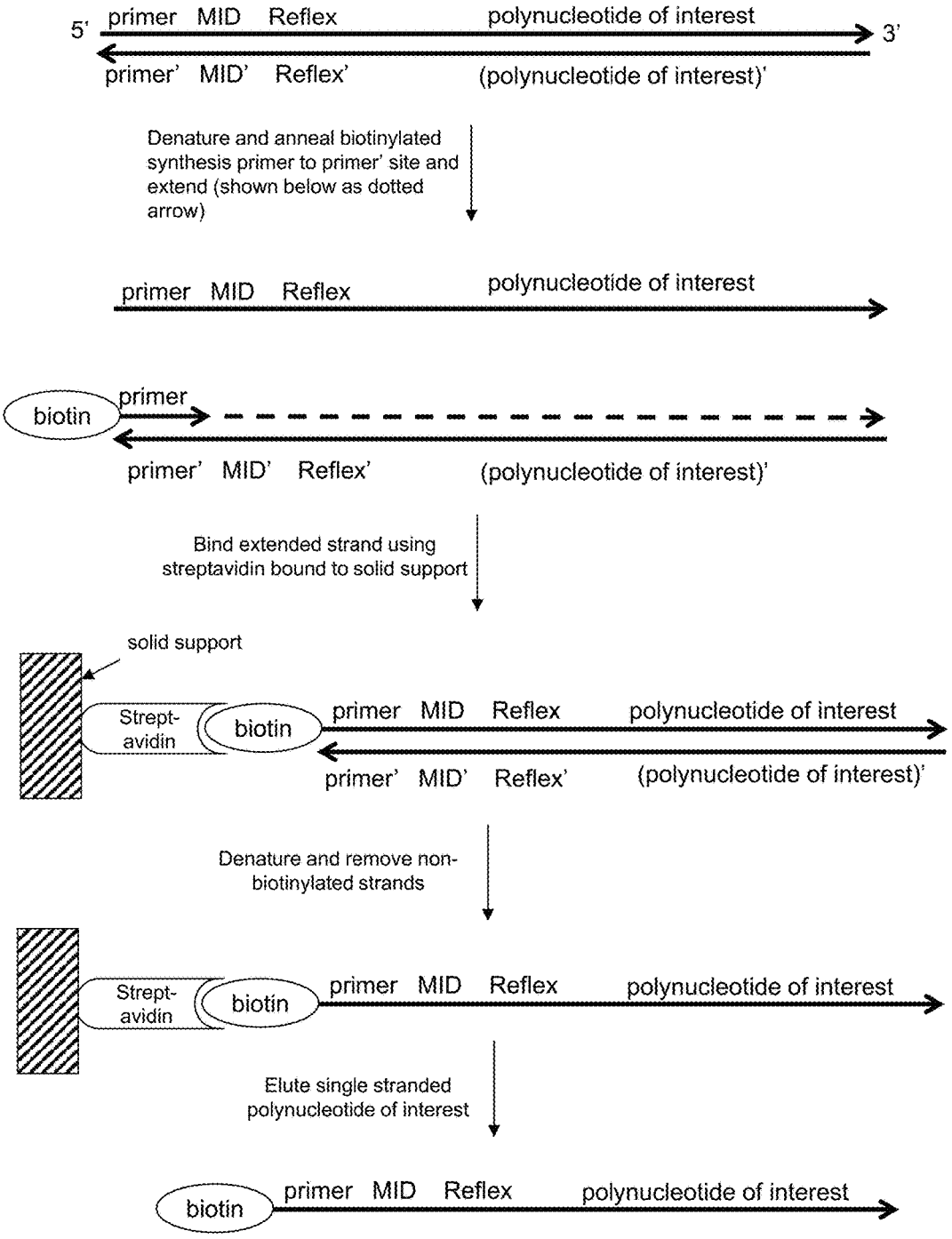
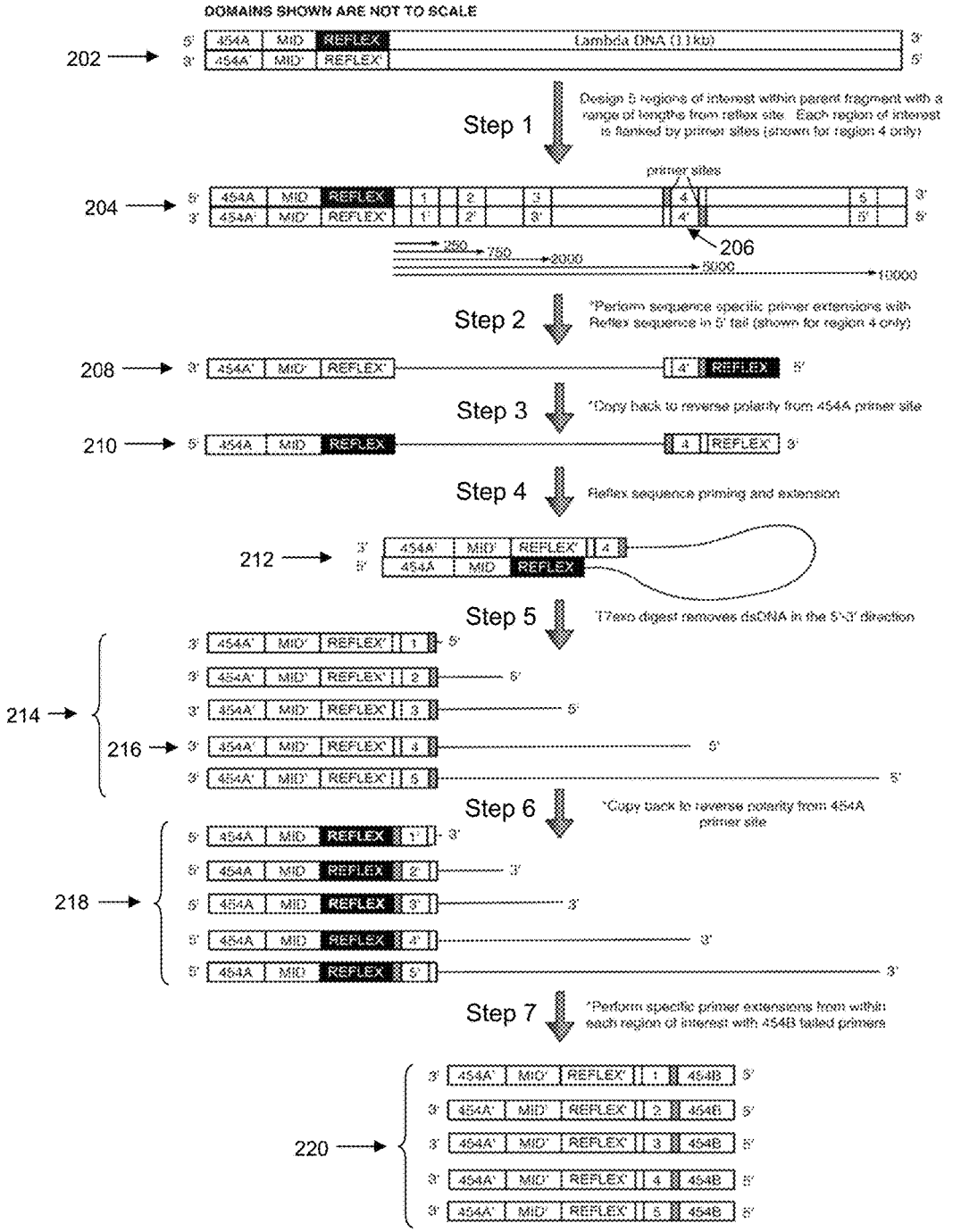

5'
primer MID Reflex
polynucleotide of interest
3'
primer' MID' Reflex'
(polynucleotide of interest)'
Denature and anneal biotinylated synthesis primer to primer' site and extend (shown below as dotted arrow)
primer MID Reflex
polynucleotide of interest
biotin
primer
primer' MID' Reflex'
(polynucleotide of interest)'
Bind extended strand using streptavidin bound to solid support
solid support
Strept-avidin
biotin
primer MID Reflex
polynucleotide of interest
primer' MID' Reflex'
(polynucleotide of interest)'
Denature and remove non-biotinylated strands
Strept-avidin
biotin
primer MID Reflex
polynucleotide of interest
Elute single stranded polynucleotide of interest
biotin
primer MID Reflex
polynucleotide of interest

Fig. 2



Primer extension reactions with * may be performed such that isolation of single strand species is facilitated (e.g., using primers with binding moieties and/or multiple cycles of extension)

Fig. 3

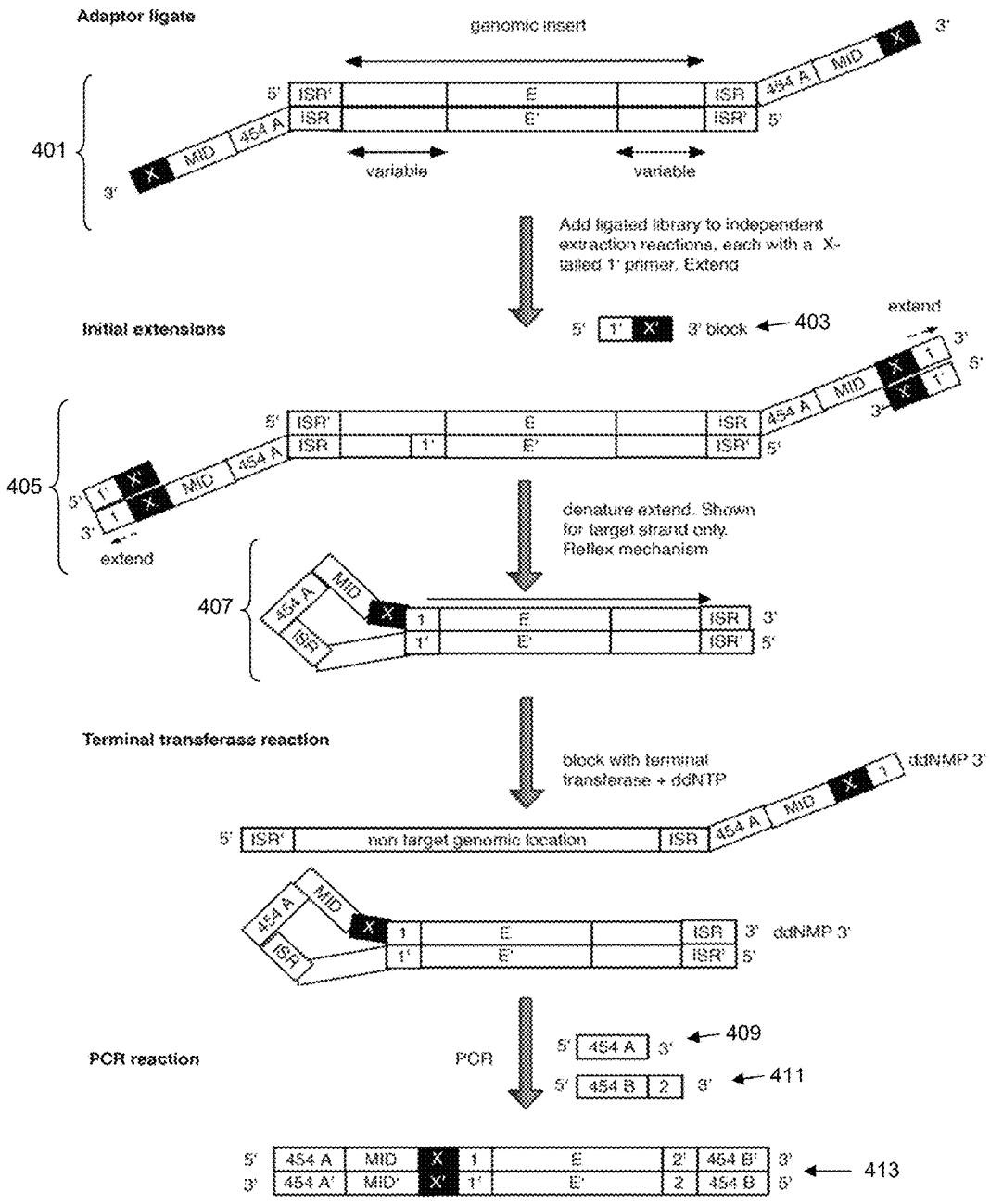

Adaptor ligate
genomic insert
401
variable
variable
Add ligated library to independent extraction reactions, each with a X-tailed 1' primer. Extend
403
Initial extensions
extend
405
extend
denature extend. Shown for target strand only. Reflex mechanism
407
Terminal transferase reaction
block with terminal transferase + ddNTP
ddNMP 3'
non target genomic location
PCR reaction
PCR
409
411
413

Fig. 4

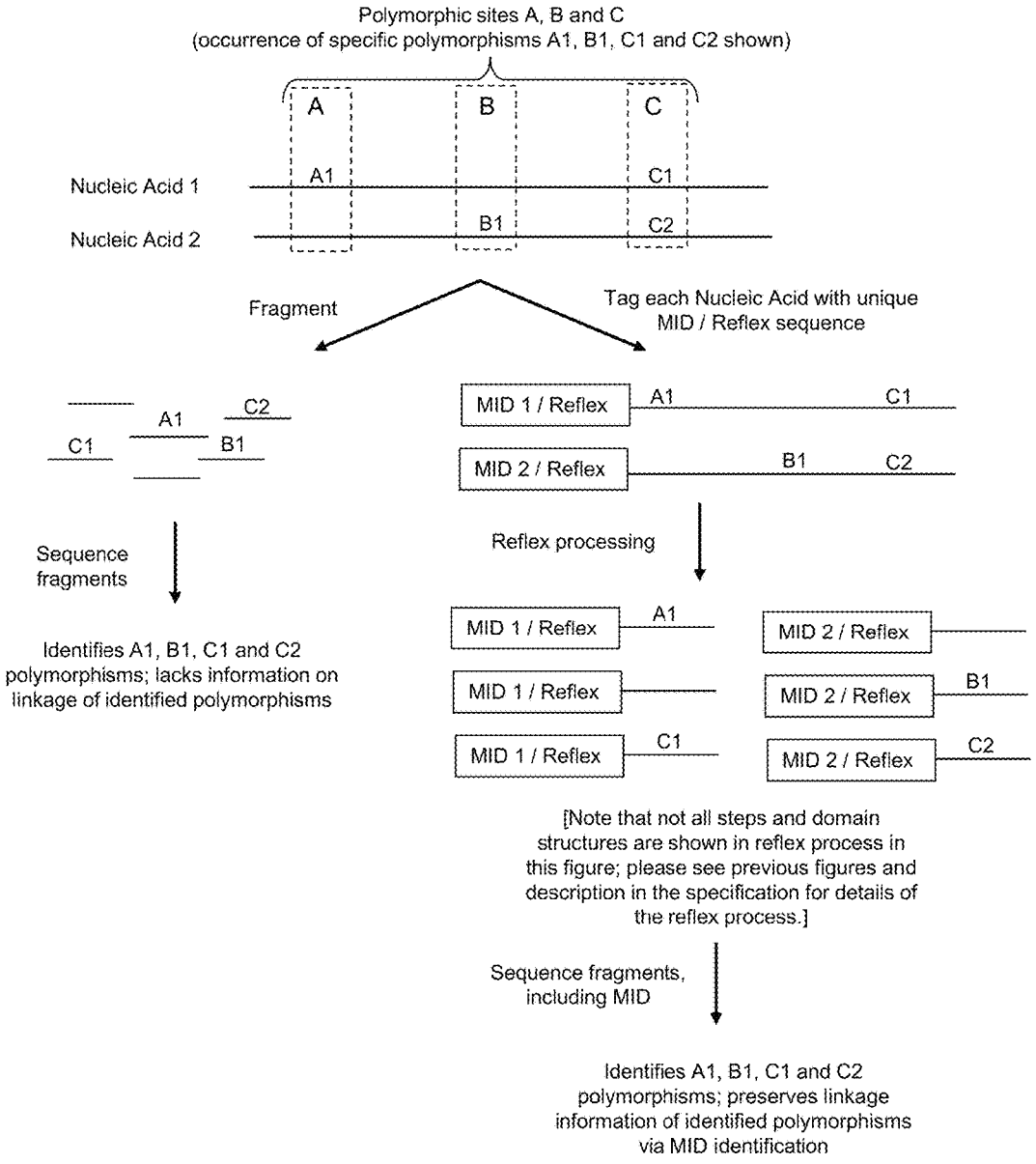
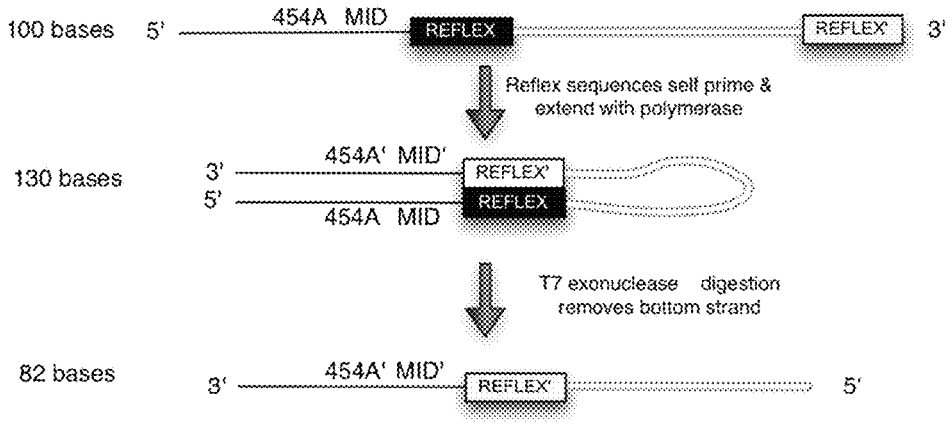
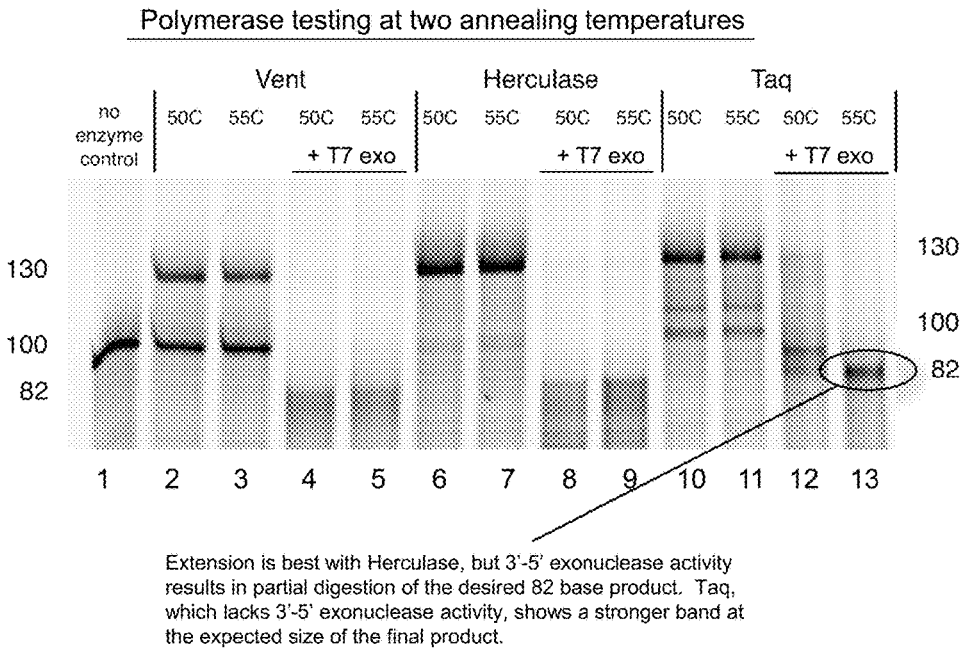

Polymorphic sites A, B and C
(occurrence of specific polymorphisms A1, B1, C1 and C2 shown)
A
B
C
Nucleic Acid 1
A1
C1
Nucleic Acid 2
B1
C2
Fragment
Tag each Nucleic Acid with unique MID / Reflex sequence
A1
C2
C1
B1
Sequence fragments
Identifies A1, B1, C1 and C2 polymorphisms; lacks information on linkage of identified polymorphisms
MID 1 / Reflex
A1
C1
MID 2 / Reflex
B1
C2
Reflex processing
MID 1 / Reflex
A1
MID 2 / Reflex
MID 1 / Reflex
MID 2 / Reflex
B1
MID 1 / Reflex
C1
MID 2 / Reflex
C2
[Note that not all steps and domain structures are shown in reflex process in this figure; please see previous figures and description in the specification for details of the reflex process.]
Sequence fragments, including MID
Identifies A1, B1, C1 and C2 polymorphisms; preserves linkage information of identified polymorphisms via MID identification

Fig. 5

A.



B.



Extension is best with Herculase, but 3'-5' exonuclease activity results in partial digestion of the desired 82 base product. Taq, which lacks 3'-5' exonuclease activity, shows a stronger band at the expected size of the final product.

Fig. 6

A.


5'
454A MID
REFLEX
REFLEX'
3'
+ Competitor
5'
REFLEX'
3'

B.

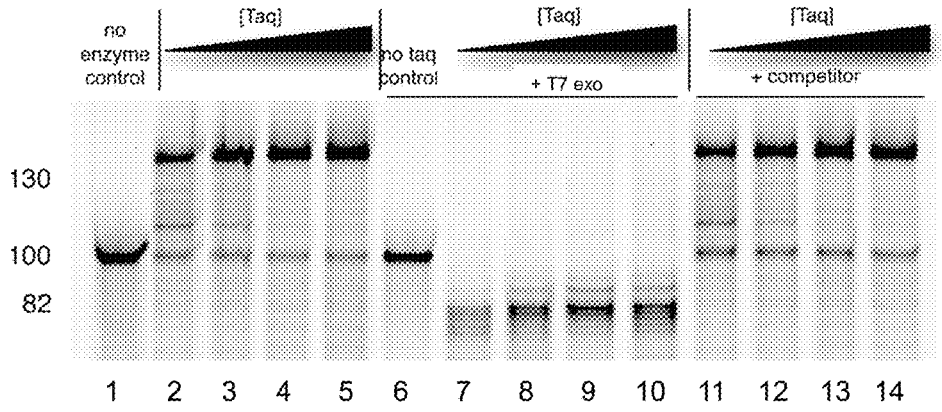
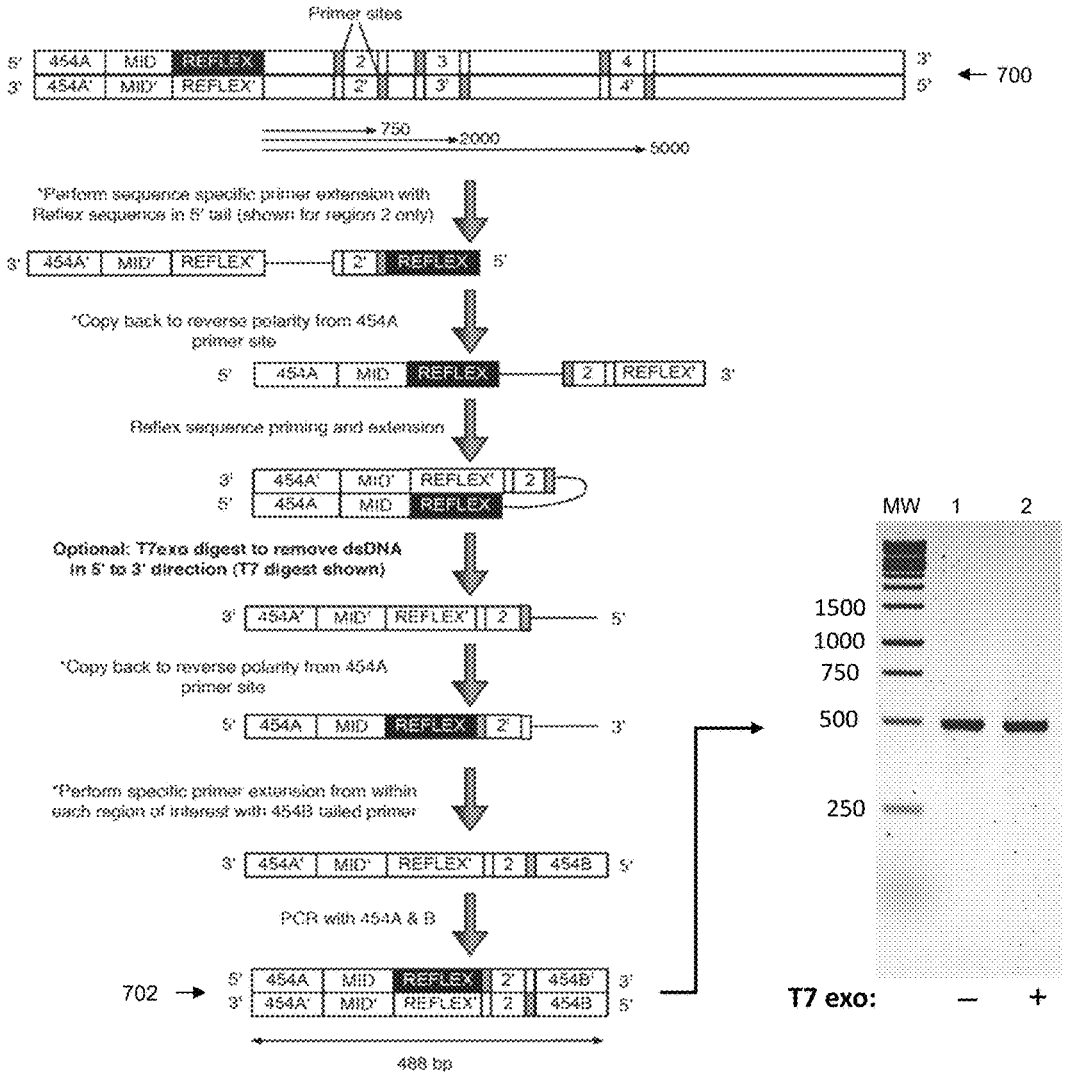

no enzyme control
[Taq]
no taq control
[Taq]
+ T7 exo
[Taq]
+ competitor
130
100
82
1 2 3 4 5 6 7 8 9 10 11 12 13 14

Fig. 7



Primer extension reactions with * may be performed such that isolation of single strand species is facilitated (e.g., using primers with binding moieties and/or multiple cycles of extension)

Fig. 8

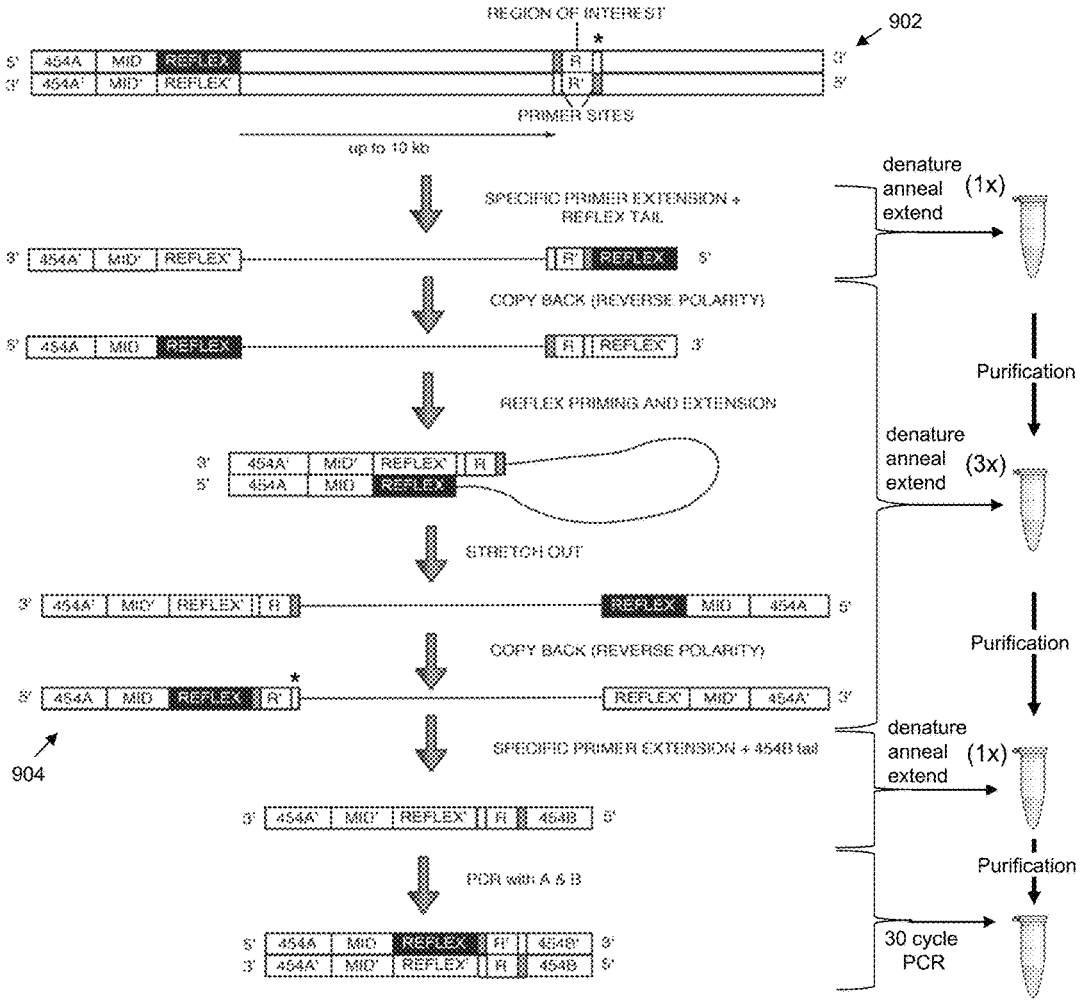

REGION OF INTEREST
902
454A
MID
REFLEX
R
PRIMER SITES
up to 10 kb
SPECIFIC PRIMER EXTENSION + REFLEX TAIL
COPY BACK (REVERSE POLARITY)
REFLEX PRIMING AND EXTENSION
STRETCH OUT
COPY BACK (REVERSE POLARITY)
SPECIFIC PRIMER EXTENSION + 454B tail
904
454B
PCR with A & B
denature
anneal
extend
(1x)
Purification
(3x)
30 cycle
PCR

Fig. 9

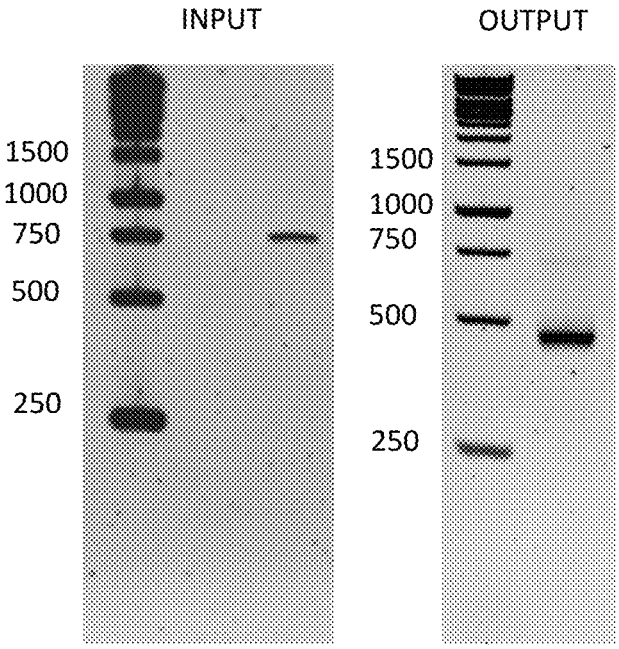
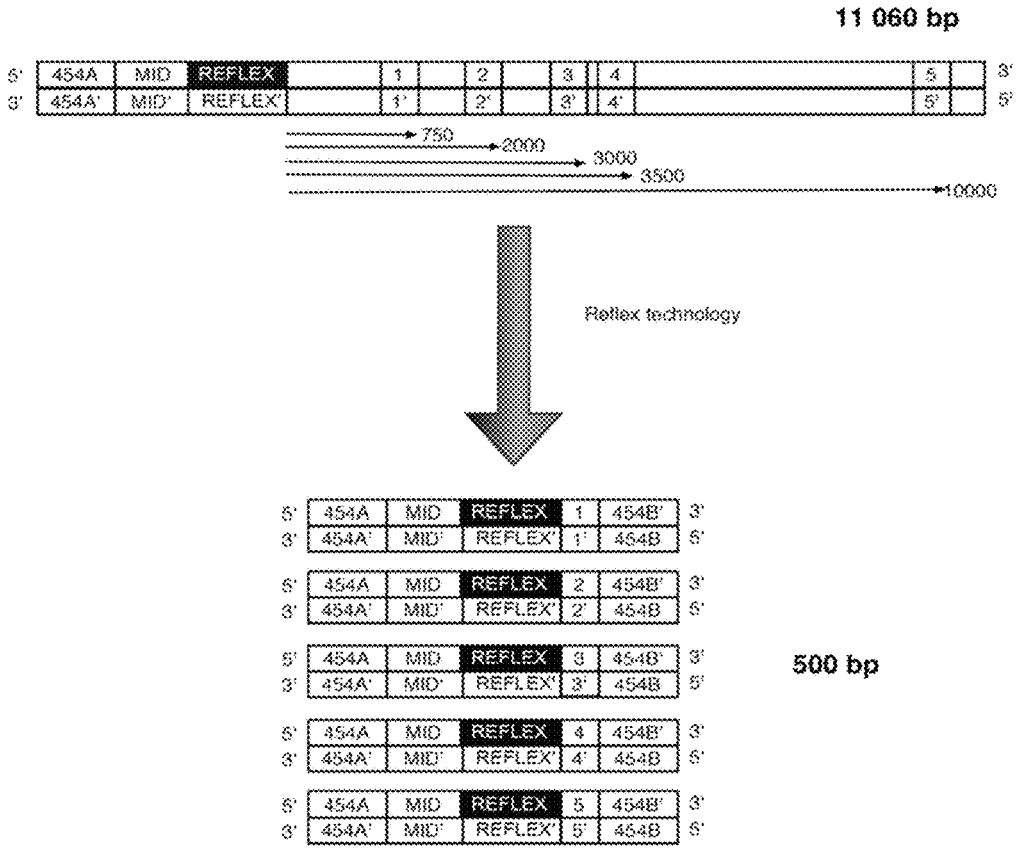


Fig. 10


11 060 bp
5' 454A MID REFLEX 1 2 3 4 5 3'
3' 454A' MID' REFLEX' 1' 2' 3' 4' 5' 5'
750
2000
3000
3500
10000
Reflex technology
5' 454A MID REFLEX 1 454B' 3'
3' 454A' MID' REFLEX' 1' 454B 5'
5' 454A MID REFLEX 2 454B' 3'
3' 454A' MID' REFLEX' 2' 454B 5'
5' 454A MID REFLEX 3 454B' 3'
3' 454A' MID' REFLEX' 3' 454B 5'
5' 454A MID REFLEX 4 454B' 3'
3' 454A' MID' REFLEX' 4' 454B 5'
5' 454A MID REFLEX 5 454B' 3'
3' 454A' MID' REFLEX' 5' 454B 5'
500 bp

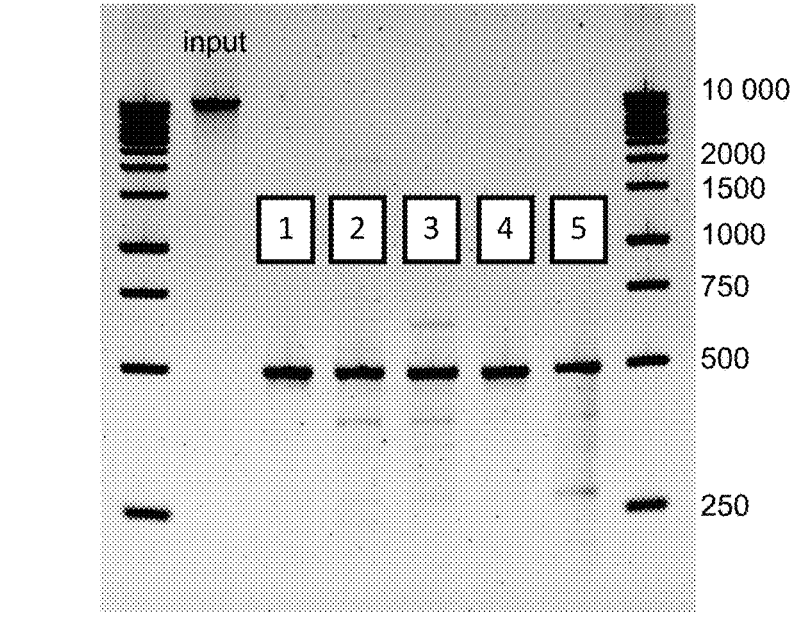
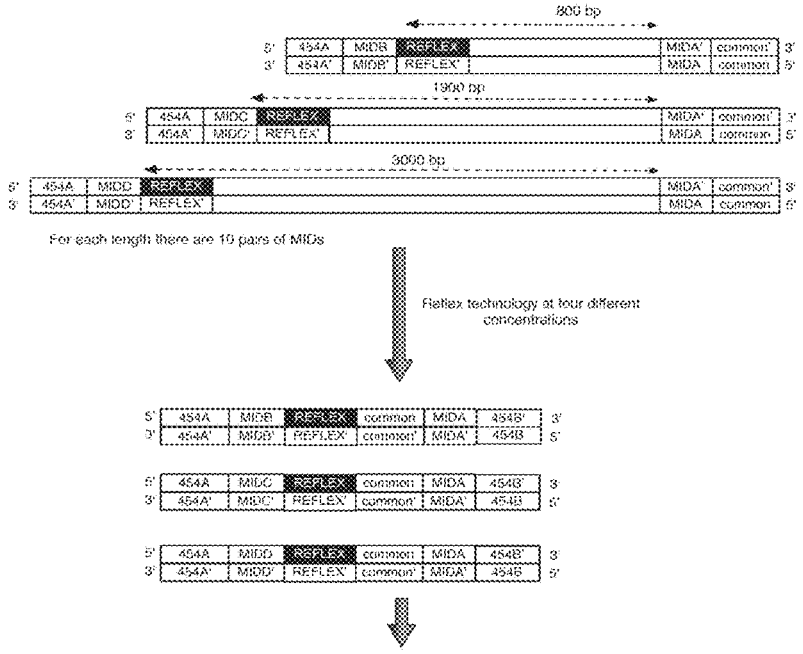

input
1
2
3
4
5
10 000
2000
1500
1000
750
500
250
Lanes: 1 2 3 4 5 6

Fig. 11


800 bp
1900 bp
3000 bp
454A
MIDB
MIDC
MIDD
REFLEX
MIDA
common
454B
For each length there are 10 pairs of MIDs
Reflex technology at four different concentrations
Roche 454 Sequencing

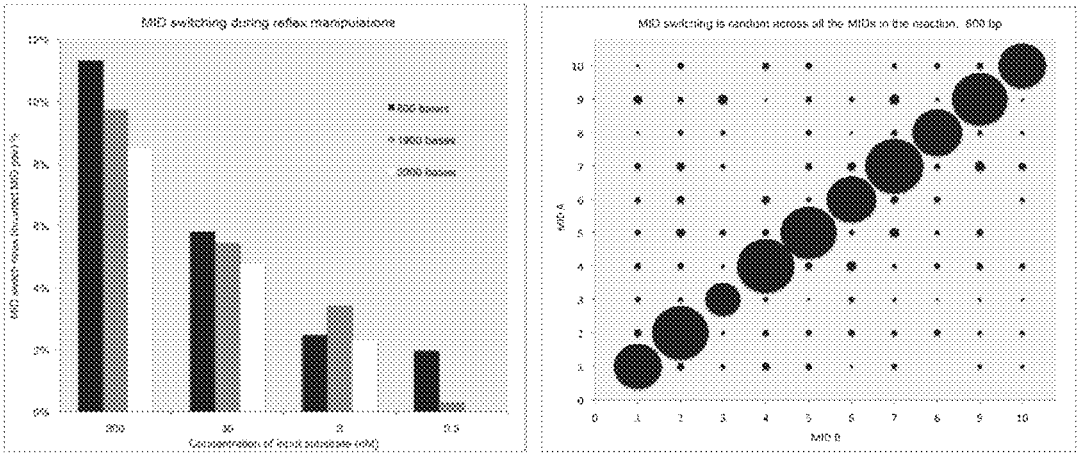

MID switching during reflex manipulations
MID switching is random across all the MIDs in the reaction. 800 bp
MID A
MID B

Fig. 12

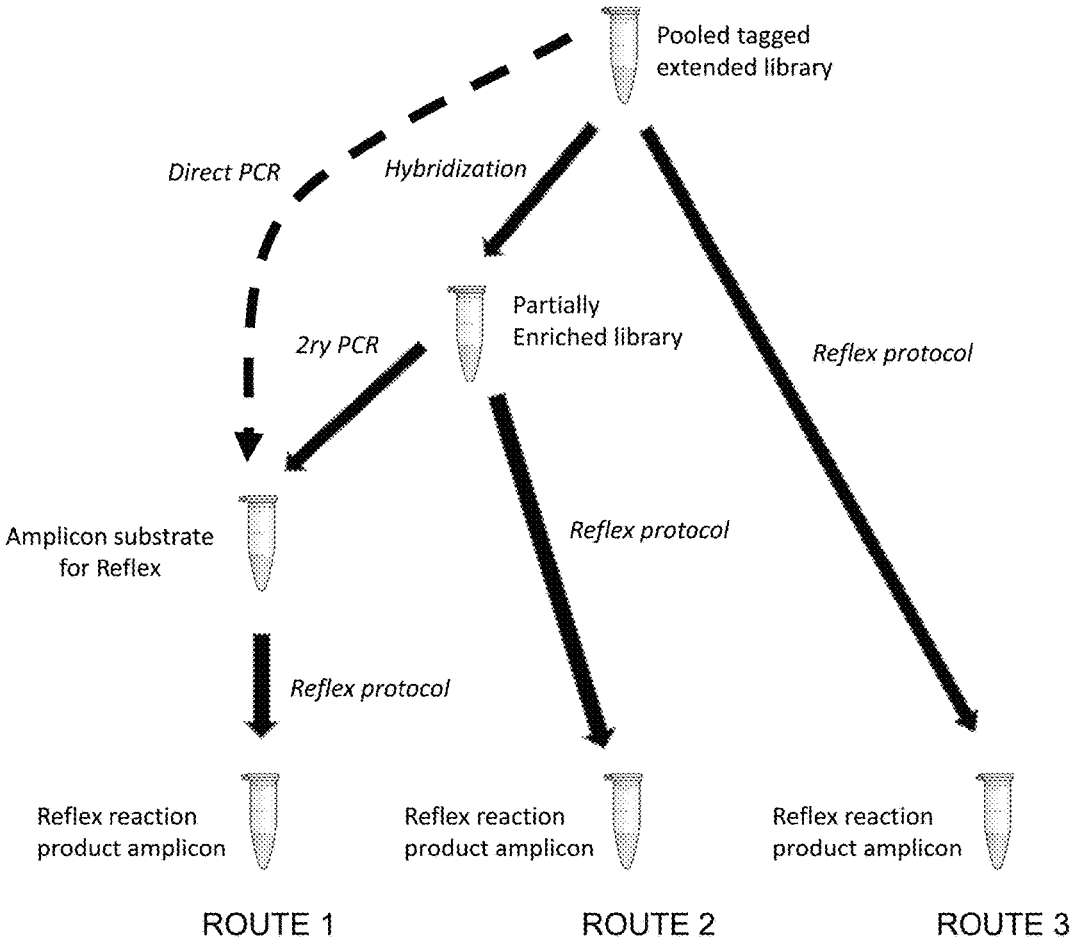

Pooled tagged extended library
Direct PCR
Hybridization
Partially Enriched library
2ry PCR
Reflex protocol
Amplicon substrate for Reflex
Reflex protocol
Reflex protocol
Reflex reaction product amplicon
Reflex reaction product amplicon
Reflex reaction product amplicon
ROUTE 1
ROUTE 2
ROUTE 3

Fig. 13

METHODS FOR ANALYZING NUCLEIC ACIDS FROM SINGLE CELLS

CROSS REFERENCE TO RELATED APPLICATIONS

This application is a continuation of U.S. application Ser. No. 15/677,957, filed Aug. 15, 2017, now patent Ser. No. 10/155,981, which is a continuation of U.S. application Ser. No. 14/792,094, filed Jul. 6, 2015, which is a continuation of U.S. application Ser. No. 14/172,694, filed Feb. 4, 2014, now U.S. Pat. No. 9,102,980, which is a continuation of U.S. application Ser. No. 14/021,790, filed Sep. 9, 2013, now U.S. Pat. No. 8,679,756, which is a continuation of U.S. application Ser. No. 13/859,450, filed Apr. 9, 2013, now U.S. Pat. No. 8,563,274, which is a continuation of U.S. application Ser. No. 13/622,872, filed Sep. 19, 2012, which is a continuation of U.S. application Ser. No. 13/387,343, filed Feb. 15, 2012, now U.S. Pat. No. 8,298,767, which is a § 371 National Phase Application of PCT/IB32010/002243, filed Aug. 13, 2010, which claims priority to U.S. Provisional Application No. 61/235,595, filed Aug. 20, 2009 and U.S. Provisional Application No. 61/288,792, filed Dec. 21, 2009; all of which are herein incorporated by reference.

BACKGROUND OF THE INVENTION

We have previously described methods that enable tagging each of a population of fragmented genomes and then combining them together to create a 'population library' that can be processed and eventually sequenced as a mixture. The population tags enable analysis software to parse the sequence reads into files that can be attributed to a particular genome in the population. One limitation of the overall process stems from limitations of existing DNA sequencing technologies. In particular, if fragments in the regions of interest of the genome are longer than the lengths that can be sequenced by a particular technology, then such fragments will not be fully analyzed (since sequencing proceeds from an end of a fragment inward). Furthermore, a disadvantage of any sequencing technology dependent on fragmentation is that sequence changes in one part of a particular genomic region may not be able to be linked to sequence changes in other parts of the same genome (e.g., the same chromosome) because the sequence changes reside on different fragments. (See FIG. **5** and its description below).

The present invention removes the limitations imposed by current sequencing technologies as well as being useful in a number of other nucleic acid analyses.

SUMMARY OF THE INVENTION

Aspects of the present invention are drawn to processes for moving a region of interest in a polynucleotide from a first position to a second position with regard to a domain within the polynucleotide, also referred to as a "reflex method" (or reflex process, reflex sequence process, reflex reaction, and the like). In certain embodiments, the reflex method results in moving a region of interest into functional proximity to specific domain elements present in the polynucleotide (e.g., primer sites and/or MID). Compositions, kits and systems that find use in carrying out the reflex processes described herein are also provided.

BRIEF DESCRIPTION OF THE DRAWINGS

The invention is best understood from the following detailed description when read in conjunction with the accompanying drawings. It is emphasized that, according to common practice, the various features of the drawings are not to scale. Indeed, the dimensions of the various features are arbitrarily expanded or reduced for clarity. Included in the drawings are the following figures:

FIG. **1**: Panel A is a schematic diagram illustrating moving a first domain from one site to another in a nucleic acid molecule using a reflex sequence. Panel B is a schematic diagram depicting the relative position of primer pairs (A_n-B_n primers) that find use in aspects of the reflex process described herein.

FIG. **2** shows an exemplary embodiment of using binding partner pairs (biotin/streptavidin) to isolate single stranded polynucleotides of interest.

FIG. **3** is a schematic diagram illustrating an exemplary embodiment for moving a primer site and a MID to a specific location in a nucleic acid of interest.

FIG. **4** shows a schematic diagram illustrating an exemplary use of the reflex process for generating a sample enriched for fragments having a region of interest (e.g., from a population of randomly fragmented and asymmetrically tagged polynucleotides).

FIG. **5** shows a comparison of methods for identifying nucleic acid polymorphisms in homologous nucleic acids in a sample (e.g., the same region derived from a chromosomal pair of a diploid cell or viral genomes/transcripts). The top schematic shows two nucleic acid molecules in a sample (1 and 2) having a different assortment of polymorphisms in polymorphic sites A, B and C (A1, B1, C1 and C2). Standard sequencing methods using fragmentation (left side) can identify the polymorphisms in these nucleic acids but do not retain linkage information. Employing the reflex process described herein to identify polymorphisms (right side) maintains linkage information.

FIG. **6**: Panel A is a schematic showing expected structures and sizes of nucleic acid species in the reflex process; Panel B is a polyacrylamide gel showing the nucleic acid species produced in the reflex process described in Example 1.

FIG. **7**: Panel A is a schematic showing the structure of the nucleic acid and competitor used in the reflex process; Panel B is a polyacrylamide gel showing the nucleic acid species produced in the reflex process described in Example 1.

FIG. **8** shows a flow chart of a reflex process (left) in which the T7 exonuclease step is optional. The gel on the right shows the resultant product of the reflex process either without the T7 exonuclease step (lane 1) or with the T7 exonuclease step (lane 2).

FIG. **9** shows an exemplary reflex process workflow with indications on the right as to where purification of reaction products is employed (e.g., using Agencourt beads to remove primer oligos).

FIG. **10** shows the starting material (left panel) and the resultant product generated (right panel) using a reflex process without using a T7 exonuclease step (as described in Example II). The reflex site in the starting material is a sequence normally present in the polynucleotide being processed (also called a "non-artificial" reflex site). This figure shows that the 755 base pair starting nucleic acid was processed to the expected 461 base pair product, thus confirming that a "non-artificial" reflex site is effective in transferring an adapter domain from one location to another in a polynucleotide of interest in a sequence specific manner.

FIG. **11** shows a schematic and results of an experiment in which the reflex process is performed on a single large initial template (a "parent" fragment) to generate five different products ("daughter" products) each having a differ-

ent region of interest (i.e., daughter products are produced having either region 1, 2, 3, 4 or 5).

FIG. **12** shows a schematic and results of experiments performed to determine the prevalence of intramolecular rearrangement during the reflex process (as desired) vs. intermolecular rearrangement (MID switching).

FIG. **13** shows a diagram of exemplary workflows for preparing material for and performing the reflex process.

DEFINITIONS

Unless otherwise defined, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. Still, certain elements are defined for the sake of clarity and ease of reference.

Terms and symbols of nucleic acid chemistry, biochemistry, genetics, and molecular biology used herein follow those of standard treatises and texts in the field, e.g. Kornberg and Baker, DNA Replication, Second Edition (W.H. Freeman, New York, 1992); Lehninger, Biochemistry, Second Edition (Worth Publishers, New York, 1975); Strachan and Read, Human Molecular Genetics, Second Edition (Wiley-Liss, New York, 1999); Eckstein, editor, Oligonucleotides and Analogs: A Practical Approach (Oxford University Press, New York, 1991); Gait, editor, Oligonucleotide Synthesis: A Practical Approach (IRL Press, Oxford, 1984); and the like.

"Amplicon" means the product of a polynucleotide amplification reaction. That is, it is a population of polynucleotides, usually double stranded, that are replicated from one or more starting sequences. The one or more starting sequences may be one or more copies of the same sequence, or it may be a mixture of different sequences. Amplicons may be produced by a variety of amplification reactions whose products are multiple replicates of one or more target nucleic acids. Generally, amplification reactions producing amplicons are "template-driven" in that base pairing of reactants, either nucleotides or oligonucleotides, have complements in a template polynucleotide that are required for the creation of reaction products. In one aspect, template-driven reactions are primer extensions with a nucleic acid polymerase or oligonucleotide ligations with a nucleic acid ligase. Such reactions include, but are not limited to, polymerase chain reactions (PCRs), linear polymerase reactions, nucleic acid sequence-based amplification (NASBAs), rolling circle amplifications, and the like, disclosed in the following references that are incorporated herein by reference: Mullis et al, U.S. Pat. Nos. 4,683,195; 4,965,188; 4,683,202; 4,800,159 (PCR); Gelfand et al, U.S. Pat. No. 5,210,015 (real-time PCR with "TAQMAN™" probes); Wittwer et al, U.S. Pat. No. 6,174,670; Kacian et al, U.S. Pat. No. 5,399,491 ("NASBA"); Lizardi, U.S. Pat. No. 5,854,033; Aono et al, Japanese patent publ. JP 4-262799 (rolling circle amplification); and the like. In one aspect, amplicons of the invention are produced by PCRs. An amplification reaction may be a "real-time" amplification if a detection chemistry is available that permits a reaction product to be measured as the amplification reaction progresses, e.g. "real-time PCR" described below, or "real-time NASBA" as described in Leone et al, Nucleic Acids Research, 26: 2150-2155 (1998), and like references. As used herein, the term "amplifying" means performing an amplification reaction. A "reaction mixture" means a solution containing all the necessary reactants for performing a reaction, which may include, but not be limited to, buffering agents to maintain pH at a selected level during a reaction, salts, co-factors, scavengers, and the like.

The term "assessing" includes any form of measurement, and includes determining if an element is present or not. The terms "determining", "measuring", "evaluating", "assessing" and "assaying" are used interchangeably and includes quantitative and qualitative determinations. Assessing may be relative or absolute. "Assessing the presence of" includes determining the amount of something present, and/or determining whether it is present or absent. As used herein, the terms "determining," "measuring," and "assessing," and "assaying" are used interchangeably and include both quantitative and qualitative determinations.

Polynucleotides that are "asymmetrically tagged" have left and right adapter domains that are not identical. This process is referred to generically as attaching adapters asymmetrically or asymmetrically tagging a polynucleotide, e.g., a polynucleotide fragment. Production of polynucleotides having asymmetric adapter termini may be achieved in any convenient manner. Exemplary asymmetric adapters are described in: U.S. Pat. Nos. 5,712,126 and 6,372,434; U.S. Patent Publications 2007/0128624 and 2007/0172839; and PCT publication WO/2009/032167; all of which are incorporated by reference herein in their entirety. In certain embodiments, the asymmetric adapters employed are those described in U.S. patent application Ser. No. 12/432,080, filed on Apr. 29, 2009, incorporated herein by reference in its entirety.

As one example, a user of the subject invention may use an asymmetric adapter to tag polynucleotides. An "asymmetric adapter" is one that, when ligated to both ends of a double stranded nucleic acid fragment, will lead to the production of primer extension or amplification products that have non-identical sequences flanking the genomic insert of interest. The ligation is usually followed by subsequent processing steps so as to generate the non-identical terminal adapter sequences. For example, replication of an asymmetric adapter attached fragment(s) results in polynucleotide products in which there is at least one nucleic acid sequence difference, or nucleotide/nucleoside modification, between the terminal adapter sequences. Attaching adapters asymmetrically to polynucleotides (e.g., polynucleotide fragments) results in polynucleotides that have one or more adapter sequences on one end (e.g., one or more region or domain, e.g., a primer site) that are either not present or have a different nucleic acid sequence as compared to the adapter sequence on the other end. It is noted that an adapter that is termed an "asymmetric adapter" is not necessarily itself structurally asymmetric, nor does the mere act of attaching an asymmetric adapter to a polynucleotide fragment render it immediately asymmetric. Rather, an asymmetric adapter-attached polynucleotide, which has an identical asymmetric adapter at each end, produces replication products (or isolated single stranded polynucleotides) that are asymmetric with respect to the adapter sequences on opposite ends (e.g., after at least one round of amplification/primer extension).

Any convenient asymmetric adapter, or process for attaching adapters asymmetrically, may be employed in practicing the present invention. Exemplary asymmetric adapters are described in: U.S. Pat. Nos. 5,712,126 and 6,372,434; U.S. Patent Publications 2007/0128624 and 2007/0172839; and PCT publication WO/2009/032167; all of which are incorporated by reference herein in their entirety. In certain embodiments, the asymmetric adapters

employed are those described in U.S. patent application Ser. No. 12/432,080, filed on Apr. 29, 2009, incorporated herein by reference in its entirety.

"Complementary" or "substantially complementary" refers to the hybridization or base pairing or the formation of a duplex between nucleotides or nucleic acids, such as, for instance, between the two strands of a double stranded DNA molecule or between an oligonucleotide primer and a primer site on a single stranded nucleic acid. Complementary nucleotides are, generally, A and T (or A and U), or C and G. Two single stranded RNA or DNA molecules are said to be substantially complementary when the nucleotides of one strand, optimally aligned and compared and with appropriate nucleotide insertions or deletions, pair with at least about 80% of the nucleotides of the other strand, usually at least about 90% to 95%, and more preferably from about 98 to 100%. Alternatively, substantial complementarity exists when an RNA or DNA strand will hybridize under selective hybridization conditions to its complement. Typically, selective hybridization will occur when there is at least about 65% complementary over a stretch of at least 14 to 25 nucleotides, preferably at least about 75%, more preferably at least about 90% complementary. See, M. Kanehisa Nucleic Acids Res. 12:203 (1984), incorporated herein by reference.

"Duplex" means at least two oligonucleotides and/or polynucleotides that are fully or partially complementary undergo Watson-Crick type base pairing among all or most of their nucleotides so that a stable complex is formed. The terms "annealing" and "hybridization" are used interchangeably to mean the formation of a stable duplex. "Perfectly matched" in reference to a duplex means that the poly- or oligonucleotide strands making up the duplex form a double stranded structure with one another such that every nucleotide in each strand undergoes Watson-Crick base pairing with a nucleotide in the other strand. A stable duplex can include Watson-Crick base pairing and/or non-Watson-Crick base pairing between the strands of the duplex (where base pairing means the forming hydrogen bonds). In certain embodiments, a non-Watson-Crick base pair includes a nucleoside analog, such as deoxyinosine, 2, 6-diaminopurine, PNAs, LNA's and the like. In certain embodiments, a non-Watson-Crick base pair includes a "wobble base", such as deoxyinosine, 8-oxo-dA, 8-oxo-dG and the like, where by "wobble base" is meant a nucleic acid base that can base pair with a first nucleotide base in a complementary nucleic acid strand but that, when employed as a template strand for nucleic acid synthesis, leads to the incorporation of a second, different nucleotide base into the synthesizing strand (wobble bases are described in further detail below). A "mismatch" in a duplex between two oligonucleotides or polynucleotides means that a pair of nucleotides in the duplex fails to undergo Watson-Crick bonding.

"Genetic locus," "locus," or "locus of interest" in reference to a genome or target polynucleotide, means a contiguous sub-region or segment of the genome or target polynucleotide. As used herein, genetic locus, locus, or locus of interest may refer to the position of a nucleotide, a gene or a portion of a gene in a genome, including mitochondrial DNA or other non-chromosomal DNA (e.g., bacterial plasmid), or it may refer to any contiguous portion of genomic sequence whether or not it is within, or associated with, a gene. A genetic locus, locus, or locus of interest can be from a single nucleotide to a segment of a few hundred or a few thousand nucleotides in length or more. In general, a locus of interest will have a reference sequence associated with it (see description of "reference sequence" below).

"Kit" refers to any delivery system for delivering materials or reagents for carrying out a method of the invention. In the context of reaction assays, such delivery systems include systems that allow for the storage, transport, or delivery of reaction reagents (e.g., probes, enzymes, etc. in the appropriate containers) and/or supporting materials (e.g., buffers, written instructions for performing the assay etc.) from one location to another. For example, kits include one or more enclosures (e.g., boxes) containing the relevant reaction reagents and/or supporting materials. Such contents may be delivered to the intended recipient together or separately. For example, a first container may contain an enzyme for use in an assay, while a second container contains probes.

"Ligation" means to form a covalent bond or linkage between the termini of two or more nucleic acids, e.g. oligonucleotides and/or polynucleotides, in a template-driven reaction. The nature of the bond or linkage may vary widely and the ligation may be carried out enzymatically or chemically. As used herein, ligations are usually carried out enzymatically to form a phosphodiester linkage between a 5' carbon of a terminal nucleotide of one oligonucleotide with 3' carbon of another oligonucleotide. A variety of template-driven ligation reactions are described in the following references, which are incorporated by reference: Whiteley et al, U.S. Pat. No. 4,883,750; Letsinger et al, U.S. Pat. No. 5,476,930; Fung et al, U.S. Pat. No. 5,593,826; Kool, U.S. Pat. No. 5,426,180; Landegren et al, U.S. Pat. No. 5,871,921; Xu and Kool, Nucleic Acids Research, 27: 875-881 (1999); Higgins et al, Methods in Enzymology, 68: 50-71 (1979); Engler et al, The Enzymes, 15: 3-29 (1982); and Namsaraev, U.S. patent publication 2004/0110213.

"Multiplex Identifier" (MID) as used herein refers to a tag or combination of tags associated with a polynucleotide whose identity (e.g., the tag DNA sequence) can be used to differentiate polynucleotides in a sample. In certain embodiments, the MID on a polynucleotide is used to identify the source from which the polynucleotide is derived. For example, a nucleic acid sample may be a pool of polynucleotides derived from different sources, (e.g., polynucleotides derived from different individuals, different tissues or cells, or polynucleotides isolated at different times points), where the polynucleotides from each different source are tagged with a unique MID. As such, a MID provides a correlation between a polynucleotide and its source. In certain embodiments, MIDs are employed to uniquely tag each individual polynucleotide in a sample. Identification of the number of unique MIDs in a sample can provide a readout of how many individual polynucleotides are present in the sample (or from how many original polynucleotides a manipulated polynucleotide sample was derived; see, e.g., U.S. Pat. No. 7,537,897, issued on May 26, 2009, incorporated herein by reference in its entirety). MIDs can range in length from 2 to 100 nucleotide bases or more and may include multiple subunits, where each different MID has a distinct identity and/or order of subunits. Exemplary nucleic acid tags that find use as MIDs are described in U.S. Pat. No. 7,544,473, issued on Jun. 6, 2009, and titled "Nucleic Acid Analysis Using Sequence Tokens", as well as U.S. Pat. No. 7,393,665, issued on Jul. 1, 2008, and titled "Methods and Compositions for Tagging and Identifying Polynucleotides", both of which are incorporated herein by reference in their entirety for their description of nucleic acid tags and their use in identifying polynucleotides. In certain embodiments, a set of MIDs employed to tag a plurality of samples need not have any particular common property (e.g., Tm, length, base composition, etc.), as the methods described herein can

accommodate a wide variety of unique MID sets. It is emphasized here that MIDs need only be unique within a given experiment. Thus, the same MID may be used to tag a different sample being processed in a different experiment. In addition, in certain experiments, a user may use the same MID to tag a subset of different samples within the same experiment. For example, all samples derived from individuals having a specific phenotype may be tagged with the same MID, e.g., all samples derived from control (or wild-type) subjects can be tagged with a first MID while subjects having a disease condition can be tagged with a second MID (different than the first MID). As another example, it may be desirable to tag different samples derived from the same source with different MIDs (e.g., samples derived over time or derived from different sites within a tissue). Further, MIDs can be generated in a variety of different ways, e.g., by a combinatorial tagging approach in which one MID is attached by ligation and a second MID is attached by primer extension. Thus, MIDs can be designed and implemented in a variety of different ways to track polynucleotide fragments during processing and analysis, and thus no limitation in this regard is intended.

"Nucleoside" as used herein includes the natural nucleosides, including 2'-deoxy and 2'-hydroxyl forms, e.g. as described in Kornberg and Baker, DNA Replication, 2nd Ed. (Freeman, San Francisco, 1992). "Analogs" in reference to nucleosides includes synthetic nucleosides having modified base moieties and/or modified sugar moieties, e.g. described by Scheit, Nucleotide Analogs (John Wiley, New York, 1980); Uhlman and Peyman, Chemical Reviews, 90: 543-584 (1990), or the like, with the proviso that they are capable of specific hybridization. Such analogs include synthetic nucleosides designed to enhance binding properties, reduce complexity, increase specificity, and the like. Polynucleotides comprising analogs with enhanced hybridization or nuclease resistance properties are described in Uhlman and Peyman (cited above); Crooke et al, Exp. Opin. Ther. Patents, 6: 855-870 (1996); Mesmaeker et al, Current Opinion in Structual Biology, 5: 343-355 (1995); and the like. Exemplary types of polynucleotides that are capable of enhancing duplex stability include oligonucleotide N3'→P5' phosphoramidates (referred to herein as "amidates"), peptide nucleic acids (referred to herein as "PNAs"), oligo-2'-O-alkylribonucleotides, polynucleotides containing C-5 propynylpyrimidines, locked nucleic acids ("LNAs"), and like compounds. Such oligonucleotides are either available commercially or may be synthesized using methods described in the literature.

"Polymerase chain reaction," or "PCR," means a reaction for the in vitro amplification of specific DNA sequences by the simultaneous primer extension of complementary strands of DNA. In other words, PCR is a reaction for making multiple copies or replicates of a target nucleic acid flanked by primer sites, such reaction comprising one or more repetitions of the following steps: (i) denaturing the target nucleic acid, (ii) annealing primers to the primer sites, and (iii) extending the primers by a nucleic acid polymerase in the presence of nucleoside triphosphates. Usually, the reaction is cycled through different temperatures optimized for each step in a thermal cycler instrument. Particular temperatures, durations at each step, and rates of change between steps depend on many factors well-known to those of ordinary skill in the art, e.g. exemplified by the references: McPherson et al, editors, PCR: A Practical Approach and PCR2: A Practical Approach (IRL Press, Oxford, 1991 and 1995, respectively). For example, in a conventional PCR using Taq DNA polymerase, a double stranded target nucleic acid may be denatured at a temperature >90° C., primers annealed at a temperature in the range 50-75° C., and primers extended at a temperature in the range 72-78° C. The term "PCR" encompasses derivative forms of the reaction, including but not limited to, RT-PCR, real-time PCR, nested PCR, quantitative PCR, multiplexed PCR, and the like. Reaction volumes range from a few hundred nanoliters, e.g. 200 nL, to a few hundred μL, e.g. 200 μL. "Reverse transcription PCR," or "RT-PCR," means a PCR that is preceded by a reverse transcription reaction that converts a target RNA to a complementary single stranded DNA, which is then amplified, e.g. Tecott et al, U.S. Pat. No. 5,168,038, which patent is incorporated herein by reference. "Real-time PCR" means a PCR for which the amount of reaction product, i.e. amplicon, is monitored as the reaction proceeds. There are many forms of real-time PCR that differ mainly in the detection chemistries used for monitoring the reaction product, e.g. Gelfand et al, U.S. Pat. No. 5,210,015 ("TAQMAN™"); Wittwer et al, U.S. Pat. Nos. 6,174,670 and 6,569,627 (intercalating dyes); Tyagi et al, U.S. Pat. No. 5,925,517 (molecular beacons); which patents are incorporated herein by reference. Detection chemistries for real-time PCR are reviewed in Mackay et al, Nucleic Acids Research, 30: 1292-1305 (2002), which is also incorporated herein by reference. "Nested PCR" means a two-stage PCR wherein the amplicon of a first PCR becomes the sample for a second PCR using a new set of primers, at least one of which binds to an interior location of the first amplicon. As used herein, "initial primers" in reference to a nested amplification reaction mean the primers used to generate a first amplicon, and "secondary primers" mean the one or more primers used to generate a second, or nested, amplicon. "Multiplexed PCR" means a PCR wherein multiple target sequences (or a single target sequence and one or more reference sequences) are simultaneously carried out in the same reaction mixture, e.g. Bernard et al, Anal. Biochem., 273: 221-228 (1999)(two-color real-time PCR). Usually, distinct sets of primers are employed for each sequence being amplified.

"Quantitative PCR" means a PCR designed to measure the abundance of one or more specific target sequences in a sample or specimen. Quantitative PCR includes both absolute quantitation and relative quantitation of such target sequences. Quantitative measurements are made using one or more reference sequences that may be assayed separately or together with a target sequence. The reference sequence may be endogenous or exogenous to a sample or specimen, and in the latter case, may comprise one or more competitor templates. Typical endogenous reference sequences include segments of transcripts of the following genes: β-actin, GAPDH, β_2-microglobulin, ribosomal RNA, and the like. Techniques for quantitative PCR are well-known to those of ordinary skill in the art, as exemplified in the following references that are incorporated by reference: Freeman et al, Biotechniques, 26: 112-126 (1999); Becker-Andre et al, Nucleic Acids Research, 17: 9437-9447 (1989); Zimmerman et al, Biotechniques, 21: 268-279 (1996); Diviacco et al, Gene, 122: 3013-3020 (1992); Becker-Andre et al, Nucleic Acids Research, 17: 9437-9446 (1989); and the like.

"Polynucleotide" or "oligonucleotide" is used interchangeably and each means a linear polymer of nucleotide monomers. Monomers making up polynucleotides and oligonucleotides are capable of specifically binding to a natural polynucleotide by way of a regular pattern of monomer-to-monomer interactions, such as Watson-Crick type of base pairing, base stacking, Hoogsteen or reverse Hoogsteen types of base pairing, wobble base pairing, or the like. As

described in detail below, by "wobble base" is meant a nucleic acid base that can base pair with a first nucleotide base in a complementary nucleic acid strand but that, when employed as a template strand for nucleic acid synthesis, leads to the incorporation of a second, different nucleotide base into the synthesizing strand. Such monomers and their internucleosidic linkages may be naturally occurring or may be analogs thereof, e.g. naturally occurring or non-naturally occurring analogs. Non-naturally occurring analogs may include peptide nucleic acids (PNAs, e.g., as described in U.S. Pat. No. 5,539,082, incorporated herein by reference), locked nucleic acids (LNAs, e.g., as described in U.S. Pat. No. 6,670,461, incorporated herein by reference), phosphorothioate internucleosidic linkages, bases containing linking groups permitting the attachment of labels, such as fluorophores, or haptens, and the like. Whenever the use of an oligonucleotide or polynucleotide requires enzymatic processing, such as extension by a polymerase, ligation by a ligase, or the like, one of ordinary skill would understand that oligonucleotides or polynucleotides in those instances would not contain certain analogs of internucleosidic linkages, sugar moieties, or bases at any or some positions.

Polynucleotides typically range in size from a few monomeric units, e.g. 5-40, when they are usually referred to as "oligonucleotides," to several thousand monomeric units. Whenever a polynucleotide or oligonucleotide is represented by a sequence of letters (upper or lower case), such as "ATGCCTG," it will be understood that the nucleotides are in 5'→3' order from left to right and that "A" denotes deoxyadenosine, "C" denotes deoxycytidine, "G" denotes deoxyguanosine, and "T" denotes thymidine, "I" denotes deoxyinosine, "U" denotes uridine, unless otherwise indicated or obvious from context. Unless otherwise noted the terminology and atom numbering conventions will follow those disclosed in Strachan and Read, Human Molecular Genetics 2 (Wiley-Liss, New York, 1999). Usually polynucleotides comprise the four natural nucleosides (e.g. deoxyadenosine, deoxycytidine, deoxyguanosine, deoxythymidine for DNA or their ribose counterparts for RNA) linked by phosphodiester linkages; however, they may also comprise non-natural nucleotide analogs, e.g. including modified bases, sugars, or internucleosidic linkages. It is clear to those skilled in the art that where an enzyme has specific oligonucleotide or polynucleotide substrate requirements for activity, e.g. single stranded DNA, RNA/DNA duplex, or the like, then selection of appropriate composition for the oligonucleotide or polynucleotide substrates is well within the knowledge of one of ordinary skill, especially with guidance from treatises, such as Sambrook et al, Molecular Cloning, Second Edition (Cold Spring Harbor Laboratory, New York, 1989), and like references.

"Primer" means an oligonucleotide, either natural or synthetic, that is capable, upon forming a duplex with a polynucleotide template, of acting as a point of initiation of nucleic acid synthesis and being extended from its 3' end along the template so that an extended duplex is formed. The sequence of nucleotides added during the extension process is determined by the sequence of the template polynucleotide. Usually primers are extended by a DNA polymerase. Primers are generally of a length compatible with their use in synthesis of primer extension products, and are usually are in the range of between 8 to 100 nucleotides in length, such as 10 to 75, 15 to 60, 15 to 40, 18 to 30, 20 to 40, 21 to 50, 22 to 45, 25 to 40, and so on, more typically in the range of between 18-40, 20-35, 21-30 nucleotides long, and any length between the stated ranges. Typical primers can be in the range of between 10-50 nucleotides long, such as 15-45, 18-40, 20-30, 21-25 and so on, and any length between the stated ranges. In some embodiments, the primers are usually not more than about 10, 12, 15, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 35, 40, 45, 50, 55, 60, 65, or 70 nucleotides in length.

Primers are usually single-stranded for maximum efficiency in amplification, but may alternatively be double-stranded. If double-stranded, the primer is usually first treated to separate its strands before being used to prepare extension products. This denaturation step is typically affected by heat, but may alternatively be carried out using alkali, followed by neutralization. Thus, a "primer" is complementary to a template, and complexes by hydrogen bonding or hybridization with the template to give a primer/template complex for initiation of synthesis by a polymerase, which is extended by the addition of covalently bonded bases linked at its 3' end complementary to the template in the process of DNA synthesis.

A "primer pair" as used herein refers to first and second primers having nucleic acid sequence suitable for nucleic acid-based amplification of a target nucleic acid. Such primer pairs generally include a first primer having a sequence that is the same or similar to that of a first portion of a target nucleic acid, and a second primer having a sequence that is complementary to a second portion of a target nucleic acid to provide for amplification of the target nucleic acid or a fragment thereof. Reference to "first" and "second" primers herein is arbitrary, unless specifically indicated otherwise. For example, the first primer can be designed as a "forward primer" (which initiates nucleic acid synthesis from a 5' end of the target nucleic acid) or as a "reverse primer" (which initiates nucleic acid synthesis from a 5' end of the extension product produced from synthesis initiated from the forward primer). Likewise, the second primer can be designed as a forward primer or a reverse primer.

"Primer site" (e.g., a sequencing primer site, and amplification primer site, etc.) as used herein refers to a domain in a polynucleotide that includes the sequence of a primer (e.g., a sequencing primer) and/or the complementary sequence of a primer. When present in single stranded form (e.g., in a single stranded polynucleotide), a primer site can be either the identical sequence of a primer or the complementary sequence of a primer. When present in double stranded form, a primer site contains the sequence of a primer hybridized to the complementary sequence of the primer. Thus, a primer site is a region of a polynucleotide that is either identical to or complementary to the sequence of a primer (when in a single stranded form) or a double stranded region formed between a primer sequence and its complement. Primer sites may be present in an adapter attached to a polynucleotide. The specific orientation of a primer site can be inferred by those of ordinary skill in the art from the structural features of the relevant polynucleotide and/or context in which it is used.

"Readout" means a parameter, or parameters, which are measured and/or detected that can be converted to a number or value. In some contexts, readout may refer to an actual numerical representation of such collected or recorded data. For example, a readout of fluorescent intensity signals from a microarray is the address and fluorescence intensity of a signal being generated at each hybridization site of the microarray; thus, such a readout may be registered or stored in various ways, for example, as an image of the microarray, as a table of numbers, or the like.

"Reflex site", "reflex sequence" and equivalents are used to indicate sequences in a polynucleotide that are employed

to move a domain intramolecularly from its initial location to a different location in the polynucleotide. The sequence of a reflex site can be added to a polynucleotide of interest (e.g., present in an adapter ligated to the polynucleotide), be based on a sequence naturally present within the polynucleotide of interest (e.g., a genomic sequence in the polynucleotide), or a combination of both. The reflex sequence is chosen so as to be distinct from other sequences in the polynucleotide (i.e., with little sequence homology to other sequences likely to be present in the polynucleotide, e.g., genomic or sub-genomic sequences to be processed). As such, a reflex sequence should be selected so as to not hybridize to any sequence except its complement under the conditions employed in the reflex processes herein described. As described later in this application, the complement to the reflex sequence is inserted on the same strand of the polynucleotide (e.g., the same strand of a double-stranded polynucleotide or on the same single stranded polynucleotide) in a particular location so as to facilitate an intramolecular binding event on such particular strand. Reflex sequences employed in the reflex process described herein can thus have a wide range of lengths and sequences. Reflex sequences may range from 5 to 200 nucleotide bases in length.

"Solid support", "support", and "solid phase support" are used interchangeably and refer to a material or group of materials having a rigid or semi-rigid surface or surfaces. In many embodiments, at least one surface of the solid support will be substantially flat, although in some embodiments it may be desirable to physically separate synthesis regions for different compounds with, for example, wells, raised regions, pins, etched trenches, or the like. According to other embodiments, the solid support(s) will take the form of beads, resins, gels, microspheres, or other geometric configurations. Microarrays usually comprise at least one planar solid phase support, such as a glass microscope slide.

"Specific" or "specificity" in reference to the binding of one molecule to another molecule, such as a labeled target sequence for a probe, means the recognition, contact, and formation of a stable complex between the two molecules, together with substantially less recognition, contact, or complex formation of that molecule with other molecules. In one aspect, "specific" in reference to the binding of a first molecule to a second molecule means that to the extent the first molecule recognizes and forms a complex with another molecule in a reaction or sample, it forms the largest number of the complexes with the second molecule. Preferably, this largest number is at least fifty percent. Generally, molecules involved in a specific binding event have areas on their surfaces or in cavities giving rise to specific recognition between the molecules binding to each other. Examples of specific binding include antibody-antigen interactions, enzyme-substrate interactions, formation of duplexes or triplexes among polynucleotides and/or oligonucleotides, biotin-avidin or biotin-streptavidin interactions, receptor-ligand interactions, and the like. As used herein, "contact" in reference to specificity or specific binding means two molecules are close enough that weak noncovalent chemical interactions, such as Van der Waal forces, hydrogen bonding, base-stacking interactions, ionic and hydrophobic interactions, and the like, dominate the interaction of the molecules.

As used herein, the term "T_m" is used in reference to the "melting temperature." The melting temperature is the temperature (e.g., as measured in ° C.) at which a population of double-stranded nucleic acid molecules becomes half dissociated into single strands. Several equations for calculating the T_m of nucleic acids are known in the art (see e.g., Anderson and Young, Quantitative Filter Hybridization, in Nucleic Acid Hybridization (1985). Other references (e.g., Allawi, H. T. & SantaLucia, J., Jr., Biochemistry 36, 10581-94 (1997)) include alternative methods of computation which take structural and environmental, as well as sequence characteristics into account for the calculation of T_m.

"Sample" means a quantity of material from a biological, environmental, medical, or patient source in which detection, measurement, or labeling of target nucleic acids is sought. On the one hand it is meant to include a specimen or culture (e.g., microbiological cultures). On the other hand, it is meant to include both biological and environmental samples. A sample may include a specimen of synthetic origin. Biological samples may be animal, including human, fluid, solid (e.g., stool) or tissue, as well as liquid and solid food and feed products and ingredients such as dairy items, vegetables, meat and meat by-products, and waste. Biological samples may include materials taken from a patient including, but not limited to cultures, blood, saliva, cerebral spinal fluid, pleural fluid, milk, lymph, sputum, semen, needle aspirates, and the like. Biological samples may be obtained from all of the various families of domestic animals, as well as feral or wild animals, including, but not limited to, such animals as ungulates, bear, fish, rodents, etc. Environmental samples include environmental material such as surface matter, soil, water and industrial samples, as well as samples obtained from food and dairy processing instruments, apparatus, equipment, utensils, disposable and non-disposable items. These examples are not to be construed as limiting the sample types applicable to the present invention.

The terms "upstream" and "downstream" in describing nucleic acid molecule orientation and/or polymerization are used herein as understood by one of skill in the art. As such, "downstream" generally means proceeding in the 5' to 3' direction, i.e., the direction in which a nucleotide polymerase normally extends a sequence, and "upstream" generally means the converse. For example, a first primer that hybridizes "upstream" of a second primer on the same target nucleic acid molecule is located on the 5' side of the second primer (and thus nucleic acid polymerization from the first primer proceeds towards the second primer).

It is further noted that the claims may be drafted to exclude any optional element. As such, this statement is intended to serve as antecedent basis for use of such exclusive terminology as "solely", "only" and the like in connection with the recitation of claim elements, or the use of a "negative" limitation.

DETAILED DESCRIPTION OF THE INVENTION

The invention is drawn to compositions and methods for intramolecular nucleic acid rearrangement that find use in various applications of genetic analysis, including sequencing, as well as general molecular biological manipulations of polynucleotide structures.

Before the present invention is described, it is to be understood that this invention is not limited to particular embodiments described, as such may, of course, vary. It is also to be understood that the terminology used herein is for the purpose of describing particular embodiments only, and is not intended to be limiting, since the scope of the present invention will be limited only by the appended claims.

Where a range of values is provided, it is understood that each intervening value, to the tenth of the unit of the lower limit unless the context clearly dictates otherwise, between the upper and lower limits of that range is also specifically

disclosed. Each smaller range between any stated value or intervening value in a stated range and any other stated or intervening value in that stated range is encompassed within the invention. The upper and lower limits of these smaller ranges may independently be included or excluded in the range, and each range where either, neither or both limits are included in the smaller ranges is also encompassed within the invention, subject to any specifically excluded limit in the stated range. Where the stated range includes one or both of the limits, ranges excluding either or both of those included limits are also included in the invention.

Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. Although any methods and materials similar or equivalent to those described herein can be used in the practice or testing of the present invention, some potential and preferred methods and materials are now described. All publications mentioned herein are incorporated herein by reference to disclose and describe the methods and/or materials in connection with which the publications are cited. It is understood that the present disclosure supersedes any disclosure of an incorporated publication to the extent there is a contradiction.

It must be noted that as used herein and in the appended claims, the singular forms "a", "an", and "the" include plural referents unless the context clearly dictates otherwise. Thus, for example, reference to "a nucleic acid" includes a plurality of such nucleic acids and reference to "the compound" includes reference to one or more compounds and equivalents thereof known to those skilled in the art, and so forth.

The practice of the present invention may employ, unless otherwise indicated, conventional techniques and descriptions of organic chemistry, polymer technology, molecular biology (including recombinant techniques), cell biology, biochemistry, and immunology, which are within the skill of the art. Such conventional techniques include polymer array synthesis, hybridization, ligation, and detection of hybridization using a label. Specific illustrations of suitable techniques can be had by reference to the example herein below. However, other equivalent conventional procedures can, of course, also be used. Such conventional techniques and descriptions can be found in standard laboratory manuals such as *Genome Analysis: A Laboratory Manual Series* (Vols. I-IV), *Using Antibodies: A Laboratory Manual*, Cells: *A Laboratory Manual, PCR Primer: A Laboratory Manual*, and *Molecular Cloning: A Laboratory Manual* (all from Cold Spring Harbor Laboratory Press), Stryer, L. (1995) *Biochemistry* (4th Ed.) Freeman, New York, Gait, "*Oligonucleotide Synthesis: A Practical Approach*" 1984, IRL Press, London, Nelson and Cox (2000), Lehninger, A., *Principles of Biochemistry* 3rd Ed., W. H. Freeman Pub., New York, N.Y. and Berg et al. (2002) *Biochemistry,* 5th Ed., W. H. Freeman Pub., New York, N.Y., all of which are herein incorporated in their entirety by reference for all purposes.

The publications discussed herein are provided solely for their disclosure prior to the filing date of the present application. Nothing herein is to be construed as an admission that the present invention is not entitled to antedate such publication by virtue of prior invention. Further, the dates of publication provided may be different from the actual publication dates which may need to be independently confirmed.

As summarized above, aspects of the present invention are drawn to the use of a 'reflex' sequence present in a polynucleotide (e.g., in an adapter structure of the polynucleotide, in a genomic region of the polynucleotide, or a combination of both) to move a domain of the polynucleotide intra-molecularly from a first location to a second location. The reflex process described herein finds use in any number of applications, e.g., placing functional elements of a polynucleotide (e.g., sequencing primer sites and/or MID tags) into proximity to a desired sub-region of interest.

Nucleic Acids

The reflex process (as described in detail below) can be employed for the manipulation and analysis of nucleic acid sequences of interest from virtually any nucleic acid source, including but not limited to genomic DNA, complementary DNA (cDNA), RNA (e.g., messenger RNA, ribosomal RNA, short interfering RNA, microRNA, etc.), plasmid DNA, mitochondrial DNA, synthetic DNA, etc. Furthermore, any organism, organic material or nucleic acid-containing substance can be used as a source of nucleic acids to be processed in accordance with the present invention including, but not limited to, plants, animals (e.g., reptiles, mammals, insects, worms, fish, etc.), tissue samples, bacteria, fungi (e.g., yeast), phage, viruses, cadaveric tissue, archaeological/ancient samples, etc. In certain embodiments, the nucleic acids in the nucleic acid sample are derived from a mammal, where in certain embodiments the mammal is a human.

In certain embodiments, the nucleic acid sequences are enriched prior to the reflex sequence process. By enriched is meant that the nucleic acid is subjected to a process that reduces the complexity of the nucleic acids, generally by increasing the relative concentration of particular nucleic acid species in the sample (e.g., having a specific locus of interest, including a specific nucleic acid sequence, lacking a locus or sequence, being within a specific size range, etc.). There are a wide variety of ways to enrich nucleic acids having a specific characteristic(s) or sequence, and as such any convenient method to accomplish this may be employed. The enrichment (or complexity reduction) can take place at any of a number of steps in the process, and will be determined by the desires of the user. For example, enrichment can take place in individual parental samples (e.g., untagged nucleic acids prior to adaptor ligation) or in multiplexed samples (e.g., nucleic acids tagged with primer sites, MID and/or reflex sequences and pooled; MID are described in further detail below).

In certain embodiments, nucleic acids in the nucleic acid sample are amplified prior to analysis. In certain of these embodiments, the amplification reaction also serves to enrich a starting nucleic acid sample for a sequence or locus of interest. For example, a starting nucleic acid sample can be subjected to a polymerase chain reaction (PCR) that amplifies one or more region of interest. In certain embodiments, the amplification reaction is an exponential amplification reaction, whereas in certain other embodiments, the amplification reaction is a linear amplification reaction. Any convenient method for performing amplification reactions on a starting nucleic acid sample can be used in practicing the subject invention. In certain embodiments, the nucleic acid polymerase employed in the amplification reaction is a polymerase that has proofreading capability (e.g., phi29 DNA Polymerase, *Thermococcus litoralis* DNA polymerase, *Pyrococcus furiosus* DNA polymerase, etc.).

In certain embodiments, the nucleic acid sample being analyzed is derived from a single source (e.g., a single organism, virus, tissue, cell, subject, etc.), whereas in other embodiments, the nucleic acid sample is a pool of nucleic acids extracted from a plurality of sources (e.g., a pool of nucleic acids from a plurality of organisms, tissues, cells, subjects, etc.), where by "plurality" is meant two or more. As

such, in certain embodiments, a nucleic acid sample can contain nucleic acids from 2 or more sources, 3 or more sources, 5 or more sources, 10 or more sources, 50 or more sources, 100 or more sources, 500 or more sources, 1000 or more sources, 5000 or more sources, up to and including about 10,000 or more sources.

In certain embodiments, nucleic acid fragments that are to be pooled with nucleic acid fragments derived from a plurality of sources (e.g., a plurality of organisms, tissues, cells, subjects, etc.), where by "plurality" is meant two or more. In such embodiments, the nucleic acids derived from each source includes a multiplex identifier (MID) such that the source from which the each tagged nucleic acid fragment was derived can be determined. In such embodiments, each nucleic acid sample source is correlated with a unique MID, where by unique MID is meant that each different MID employed can be differentiated from every other MID employed by virtue of at least one characteristic, e.g., the nucleic acid sequence of the MID. Any type of MID can be used, including but not limited to those described in co-pending U.S. patent application Ser. No. 11/656,746, filed on Jan. 22, 2007, and titled "Nucleic Acid Analysis Using Sequence Tokens", as well as U.S. Pat. No. 7,393,665, issued on Jul. 1, 2008, and titled "Methods and Compositions for Tagging and Identifying Polynucleotides", both of which are incorporated herein by reference in their entirety for their description of nucleic acid tags and their use in identifying polynucleotides. In certain embodiments, a set of MIDs employed to tag a plurality of samples need not have any particular common property (e.g., T_m, length, base composition, etc.), as the asymmetric tagging methods (and many tag readout methods, including but not limited to sequencing of the tag or measuring the length of the tag) can accommodate a wide variety of unique MID sets.

In certain embodiments, each individual polynucleotide (e.g., double-stranded or single-stranded, as appropriate to the methodological details employed) in a sample to be analyzed is tagged with a unique MID so that the fate of each polynucleotide can be tracked in subsequent processes (where, as noted above, unique MID is meant to indicate that each different MID employed can be differentiated from every other MID employed by virtue of at least one characteristic, e.g., the nucleic acid sequence of the MID). For example (and as described below), having each nucleic acid tagged with a unique MID allows analysis of the sequence of each individual nucleic acid using the reflex sequence methods described herein. This allows the linkage of sequence information for large nucleic acid fragments that cannot be sequenced in a single sequencing run.

Reflex Sequence Process

As summarized above, aspects of the present invention include methods and compositions for moving a domain in a polynucleotide from a first location to a second location in the polynucleotide. An exemplary embodiment is shown in FIG. **1**A.

FIG. **1**A shows a single stranded polynucleotide **100** comprising, in a 5' to 3' orientation, a first domain (**102**; the domain to be moved); a reflex sequence **104**; a nucleic acid sequence **106** having a site distal to the first domain (Site A), and a complement of the reflex sequence **108** (positioned at the 3' terminus of the polynucleotide). The steps of the reflex method described below will move the first domain into closer proximity to Site A. It is noted here that the prime designation in FIG. **1**A denotes a complementary sequence of a domain. For example, First Domain' is the complement of the First Domain.

In Step **1**, the reflex sequence and its complement in the polynucleotide are annealed intramolecularly to form polynucleotide structure **112**, with the polynucleotide folding back on itself and hybridizing to form a region of complementarity (i.e., double stranded reflex/reflex' region). In this configuration, the 3' end of the complement of the reflex sequence can serve as a nucleic acid synthesis priming site. Nucleic acid synthesis from this site is then performed in extension Step **2** producing a complement of the first domain at the 3' end of the nucleic acid extension (shown in polynucleotide **114**; extension is indicated with dotted arrow labeled "extend").

Denaturation of polynucleotide **114** (e.g., by heat) generates linear single stranded polynucleotide **116**. As shown in FIG. **1**, resultant polynucleotide **116** contains a complement of the first domain at a position proximal to Site A (i.e., separated by only the complement of the reflex sequence). This resultant polynucleotide may be used for any subsequent analysis or processing steps as desired by the user (e.g., sequencing, as a template for amplification (linear, PCR, etc.), sequence specific extraction, etc.).

In alternative embodiments, the first domain and reflex sequence are removed from the 5' end of the double-stranded region of polynucleotide **114** (shown in polynucleotide **118**; removal is shown in the dotted arrow labeled "remove"). Removal of this region may be accomplished by any convenient method, including, but not limited to, treatment (under appropriate incubation conditions) of polynucleotide structure **114** with T7 exonuclease or by treatment with Lambda exonuclease; the Lambda exonuclease can be employed so long as the 5' end of the polynucleotide is phosphorylated. If the region is removed enzymatically, resultant polynucleotide **118** is used in place of polynucleotide **116** in subsequent steps (e.g., copying to reverse polarity).

In certain embodiments, polynucleotide **116** or **118** is used as a template to produce a double stranded polynucleotide, for example by performing a nucleic acid synthesis reaction with a primer that primes in the complement of the first domain. This step is sometimes referred to as copying to reverse polarity of a single stranded polynucleotide, and in some instances, the double-stranded intermediate product of this copying is not shown (see, e.g., FIG. **3**). For example, copying to reverse the polarity of polynucleotide **116** results in single-stranded polynucleotide **120** having, in a 5' to 3' orientation, the first domain (**122**); the reflex sequence (**124**); the complement of polynucleotide **106** (oriented with the complement of Site A (Site A'; **126**) proximal to the reflex sequence); the complement of the reflex sequence (**128**); and the complement of the first domain (**130**).

In certain embodiments, the first domain in the polynucleotide comprises one or more elements that find use in one or more subsequent processing or analysis steps. Such sequences include, but are not limited to, restriction enzyme sites, PCR primer sites, linear amplification primer sites, reverse transcription primer sites, RNA polymerase promoter sites (such as for T7, T3 or SP6 RNA polymerase), MID tags, sequencing primer sites, etc. Any convenient element can be included in the first domain and, in certain embodiments, is determined by the desires of the user of the methods described herein.

As an exemplary embodiment, suppose we want to sequence a specific polynucleotide region from multiple genomes in a pooled sample where the polynucleotide region is too long to sequence in a single reaction. For example, sequencing a polynucleotide region that is 2 kilobases or more in length using Roche 454 (Branford, Conn.)

technology, in which the length of a single sequencing run is about 400 bases. In this scenario, we can design a set of left hand primers (A_n) and right hand primers (B_n) specific for the polynucleotide region that are positioned in such a way that we can obtain direct sequences of all parts of the insert, as shown in FIG. **1**B. Note that the polynucleotide shown in FIG. **1**B (**140**) has a domain (**142**) containing a primer site and an MID denoting from which original sample(s) the polynucleotide is derived. Site 142 thus represents an example of a First Domain site such identified as **122** in the FIG. **1**A. The polynucleotide also includes a reflex site (**144**), which can be part of the polynucleotide region itself (e.g., a genomic sequence), added in a ligated adapter domain along with the primer site and the MID (an artificial sequence), or a combination of both (a sequence spanning the adapter/polynucleotide junction).

It is noted here that polynucleotide **140** can be categorized as a precursor to polynucleotide **100** in FIG. **1**A, as it does not include a 3' reflex sequence complementary to the reflex site (domain **108** in FIG. **1**A). As detailed below, polynucleotide **140** can be converted to a polynucleotide having the structural configuration of polynucleotide **100**, a polynucleotide suitable as a substrate for the reflex process described herein (e.g., by primer extension using a B_n primer and reversal of polarity).

In an exemplary embodiment, each A_n-B_n primer pair defines a nucleic acid region that is approximately 400 bases in length or less. This size range is within the single-sequencing run read length of the current Roche 454 sequencing platform; a different size range for the defined nucleic acid region may be utilized for a different sequencing platform. Thus, each product from each reflex process can be sequenced in a single run. It is noted here that primer pairs as shown in FIG. **1**B can be used to define regions 1 to 5 shown in FIG. **3** (described in further detail below).

In certain embodiments, to obtain the first part of the sequence of the polynucleotide region (i.e., in the original structure, that part of the polynucleotide closest to the first domain), we only need a right hand primer (e.g., B_0) and we do not need to transfer the MID as it is within reach of this sequencing primer (i.e., the MID is within 400 bases of sequencing primer B_0). All other B_n primers have the reflex sequence added to their 5' ends ("R" element shown on B primers) so that they read 5' reflex-B_n. However, in certain embodiments, the B_0 primer does include the reflex sequence and is used in the reflex process (along with a corresponding A_0 primer) as detailed below.

As described above, we obtain a single stranded polynucleotide having, in the 5' to 3' orientation, a primer site (e.g., for Roche 454 sequencing), an MID, a reflex sequence and the polynucleotide to be sequenced. Numerous methods for obtaining single-stranded polynucleotides of interest have been described and are known in the art, including in U.S. Pat. No. 7,217,522, issued on May 15, 2007; U.S. patent application Ser. No. 11/377,462, filed on Mar. 16, 2006; and U.S. patent application Ser. No. 12/432,080, filed on Apr. 29, 2009; each of which is incorporated by reference herein in their entirety. For example, a single stranded product can be produced using linear amplification with a primer specific for the primer site of the template. In certain embodiments, the primer includes a binding moiety to facilitate isolation of the single stranded nucleic acid of interest, e.g., to immobilize the top strand on a binding partner of the binding moiety immobilized on a solid support. Removal of a hybridized, non-biotinylated strand by denaturation using heat or high pH (or any other convenient method) serves to isolate the biotinylated strand. Binding moieties and their corresponding binding partners are sometimes referred to herein as binding partner pairs. Any convenient binding partner pairs may be used, including but not limited to biotin/avidin (or streptavidin), antigen/antibody pairs, etc.

It is noted here that while the figures and description of the reflex process provided herein depict manipulations with regard to a single stranded polynucleotide, it is not necessarily required that the single stranded polynucleotide described or depicted in the figures be present in the sample in an isolated form (i.e., isolated from its complementary strand). In other words, double stranded polynucleotides may be used where only one strand is described/depicted, which will generally be determined by the user.

The implementation of a single strand isolation step using the methods described above or variations thereof (or any other convenient single strand isolation step) will generally be based on the desires of the user. One example of isolating single stranded polynucleotides is shown in FIG. **2**. In this Figure, a starting double stranded template (with 5' to 3' orientation shown as an arrow) is denatured and primed with a biotinylated synthesis primer specific for the primer site. After extension of the primer (i.e., nucleic acid synthesis), the sample is contacted with a solid support having streptavidin bound to it. The biotin moiety (i.e., the binding partner of streptavidin) on the extended strands will bind to the solid-phase streptavidin. Denaturation and washing is then performed to remove all non-biotinylated polynucleotide strands. If desired, the bound polynucleotide, which can be used in subsequent reflex process steps (e.g., as a template for B_n primer extension reactions), may be eluted from the streptavidin support. Alternatively, the bound polynucleotide may be employed in subsequent steps of the desired process while still bound to the solid support (e.g., in solid phase extension reactions using B_n primers). This process, with minor variations depending on the template being used and the identity of the desired single stranded polynucleotide, may be employed at any of a number of steps in which a single stranded product is to be isolated. It is noted that in certain embodiments, substrate bound biotinylated polynucleotide can be used to produce and isolate non-biotinylated single stranded products (i.e., by eluting the non-biotinylated products while leaving the biotinylated templates bound to the streptavidin on the solid support). Thus, the specifics of how binding partners are used to isolate single stranded polynucleotides of interest will vary depending on experimental design parameters.

Additional single-stranded isolation/production methods include asymmetric PCR, strand-specific enzymatic degradation, and the use of in-vitro transcription followed by reverse transcriptase (IVT-RT) with subsequent destruction of the RNA strand. As noted above, any convenient single stranded production/isolation method may be employed.

To the single stranded polynucleotide shown in FIG. **1**B we anneal one of the B_n primers having the appended reflex sequence, denoted with a capital "R" (e.g., B_1) and extend the primer under nucleic acid synthesis conditions to produce a copy of the polynucleotide that has a reflex sequence at its 5' end. A single stranded copy of this polynucleotide is then produced to reverse polarity using a primer specific for the primer site in the first domain' (complement of the first domain **102**). The resulting nucleic acid has structure **100** shown in FIG. **1**A, where the first domain **102** includes the primer site and the MID. Site A (**110**) in FIG. **1** is determined by the specificity of the 5' reflex-B_n primer used.

The reflex process (e.g., as shown in FIG. **1**) is then performed to produce a product in which the primer site and

the MID are now in close proximity to the desired site (or region of interest (ROI)) within the original polynucleotide (i.e., the site defined by the primer used, e.g., B_1). The resulting polynucleotide can be used in subsequent analyses as desired by the user (e.g., Roche 454 sequencing technology).

It is noted here that, while not shown in FIGS. **1**A and **1**B, any convenient method for adding adapters to a polynucleotide to be processed as described herein may be used in the practice of the reflex process (adapters containing, e.g., primer sites, polymerase sites, MIDs, restriction enzyme sites, and reflex sequences). For example, adapters can be added at a particular position by ligation. For double stranded polynucleotides, an adapter can be configured to be ligated to a particular restriction enzyme cut site. Where a single stranded polynucleotide is employed, a double stranded adapter construct that possesses an overhang configured to bind to the end of the single-stranded polynucleotide can be used. For example, in the latter case, the end of a single stranded polynucleotide can be modified to include specific nucleotide bases that are complementary to the overhang in the double stranded adaptor using terminal transferase and specific nucleotides. In other embodiments, PCR or linear amplification methods using adapter-conjugated primers is employed to add an adapter at a site of interest. Again, any convenient method for producing a starting polynucleotide may be employed in practicing the methods of the subject invention.

In certain embodiments, the nucleic acid may be sequenced directly using a sequencing primer specific for the primer site. This sequencing reaction will read through the MID and desired site in the insert.

In certain embodiments, the polynucleotide may be isolated (or fractionated) using an appropriate A_n primer (e.g., when using B_1 as the first primer, primer A_1 can be used). In certain embodiments, the A_n primed polynucleotide is subjected to nucleic acid synthesis conditions to produce a copy of the fragment produced in the reflex process. In certain of these embodiments, the A_n primer has appended on its 5' end a primer site that can be used in subsequent steps, including sequencing reactions. Providing a primer site in the A_n primer allows amplifying and/or sequencing from both ends of the resultant fragment: from the primer site in the first domain **102** and the primer site in the A_n primer (not shown in FIG. **1**B). Because of the position of the primer sites and their distance apart (i.e., less than one sequencing run apart), sequencing from both ends will usually capture the sequence of the desired site (or ROI) and the sequence of the MID, which can be used for subsequent bioinformatic analyses, e.g., to positively identify the sample of origin. It is noted here that while sequencing in both directions is possible, it is not necessary, as sequencing from either primer site alone will capture the sequence of the ROI as well as its corresponding MID sequence.

Note that in certain embodiments, the first fragment obtained by amplification/extension from primer B_0 directly, the polarity of the ROI in the resulting fragment is reversed as compared to the ROI in fragments obtained by primers B_1-B_n. This is because the B_0-generated fragment, unlike the B_1-B_n generated fragments, has not been subjected to a reflex process which reverses the orientation of the ROI sequence with respect to the first domain/reflex sequence (as described above). Therefore, the B_0 primer may have appended to it a primer site (e.g., at its 5' end) that can be used for subsequent amplification and/or sequencing reactions (e.g., in Roche 454 sequencing system) rather than a reflex sequence as with primers B_1-B_n. However, in certain embodiments, as noted above, the reflex process may be used with a corresponding B_0-A_0 primer pair as described above, i.e., using a B_0 primer having a 5' reflex sequence and a corresponding A_0 primer with its corresponding 5' adapter domain (e.g., a primer site).

It is noted here that because the particular sections of sequence to be analyzed are defined by the A_n-B_n primer pairs (as shown and described above), a much higher sequence specificity is achieved as compared to using previous extraction methods that employ only a single oligo binding event (e.g., using probes on a microarray).

FIG. **3** provides a detailed flow chart for an exemplary embodiment that employs reflex sequences for use in sequencing multiple specific regions in a polynucleotide (i.e., regions 1, 2, 3, 4 and 5 in an 11 kb region of lambda DNA).

A single parent DNA fragment **202** is generated that includes adapter domains (i.e., a Roche 454 sequencing primer site, a single MID, and a reflex sequence) and the sequence of interest. In the example shown, the sequence of interest is from lambda DNA and the reflex sequence is present on the top strand (with its complement shown in the bottom strand). Any convenient method for producing this parent DNA fragment may be used, including amplification with a primer that includes the adapter domains (e.g., using PCR), cloning the fragment into a vector that includes the adapter domains (e.g., a vector with the adapter domains adjacent to a cloning site), or by attaching adapters to polynucleotide fragments (e.g., fragment made by random fragmentation, by sequence-specific restriction enzyme digestion, or combinations thereof). While only a single fragment with a single MID is shown, the steps in FIG. **3** are applicable to samples having multiple different fragments each with a different MID, e.g., a sample having a population of homologous fragments from any number of different sources (e.g., different individuals). FIG. **3** describes the subsequent enzymatic steps involved in creating the five daughter fragments in which regions 1, 2, 3, 4 and 5 (shown in polynucleotide **204**) are rearranged to be placed within a functional distance of the adapter domains (i.e., close enough to the adapter domains to be sequenced in a single Roche 454 sequencing reaction). Note that certain steps are shown for region 4 only (**206**).

In step **1**, the five regions of interest are defined within the parent fragment (labeled 1 to 5 in polynucleotide **204**) and corresponding primer pairs are designed for each. The distance of each region of interest from the reflex sequence is shown below polynucleotide **204**. The primer pairs are designed as described and shown in FIG. **1**B (i.e., the A_n-B_n primer pairs). For clarity, only primer sites for region 4 are shown in FIG. **3** ("primer sites" surrounding region 4). In step **2**, sequence specific primer extensions are performed (only region 4 is shown) with corresponding B_n primers to produce single stranded polynucleotides having structure **208** (i.e., having the reflex sequence on the 5' terminus). As shown, the B_n primer for region 4 will include a sequence specific primer site that primes at the 3'-most primer site noted for region 4 (where "3'-most" refers to the template strand, which in FIG. **3** is the top strand). This polynucleotide is copied back to produce polynucleotide **210** having reversed polarity (e.g., copied using a primer that hybridizes to the 454A' domain). Polynucleotide **210** has structure similar to polynucleotide **100** shown at the top of FIG. **1**. Step **4** depicts the result of the intramolecular priming between the reflex sequence and its complement followed by extension to produce the MID' and 454A' structures at the 3' end (polynucleotide **212**). In the embodiments shown in

FIG. **3**, polynucleotide **212** is treated with T7 exonuclease to remove double stranded DNA from the 5' end (as indicated above, this step is optional). The polynucleotide formed for region 4 is shown as **216** with polynucleotides for the other regions also shown (**214**).

It is noted here that the formation of each of the polynucleotides **214** may be accomplished either in separate reactions (i.e., structure with region 1 in proximity to the adapter domains is in a first sample, the structure with region 2 in proximity to the adapter region is in a second sample, etc.) or in one or more combined sample.

In step **6** the polynucleotides **214** are copied to reverse polarity to form polynucleotides **218**. In step **7**, each of these products are then primed with the second primer of the specific primer pair (see A_n primers as shown in FIG. **1**B) each having a second Roche 454 primer site (454B) attached at the 5' end, and extended to form products **220**. Steps **6** and **7** may be combined (e.g., in a single PCR or other amplification reaction).

In summary, FIG. **3** shows how the reflex process can be employed to produce five daughter fragments **220** of similar length (e.g., ~500 bp) each of which contain DNA sequences that differ in their distance from the reflex sequence in the starting structure **202** while maintaining the original MID.

FIG. **4** shows another exemplary use of the reflex process as described herein. In the embodiment shown in FIG. **4**, a target sequence (i.e., containing region of interest "E") is enriched from a pool of adapter-attached fragments. In certain embodiments, the fragments are randomly sheared, selected for a certain size range (e.g., DNA having a length from 100 to 5000 base pairs), and tagged with adapters (e.g., asymmetric adapters, e.g., as described in U.S. patent application Ser. No. 12/432,080, filed on Apr. 29, 2009). The asymmetric adaptor employed in FIG. **4** contains a sequencing primer site (454A, as used in the Roche 454 sequencing platform), an MID, an X sequence, and an internal stem region (ISR), which denotes the region of complementarity for the asymmetric adapter that is adjacent to the adapter attachment site (see, e.g., the description in U.S. application Ser. No. 12/432,080, filed on Apr. 29, 2009, incorporated herein by reference in its entirety). The X sequence can be any sequence that can serve as a binding site for a polynucleotide containing the complement of the X sequence (similar to a primer site). As described below, the X sequence allows for the annealing of an oligonucleotide having a 5' overhang that can serve as a template for extension of the 3' end of the adaptor oligonucleotide. The sequencing direction of the sequencing primer site (454A primer site in structure **401** of FIG. **4**) is oriented such that amplification of the adapter ligated fragment using the sequencing primer site proceeds away from the ligated genomic insert. This has the effect of making the initial asymmetric adapter ligated library 'inert' to amplification using this primer, e.g., in a PCR reaction.

To extract a region of interest (the "E" region), the library is mixed with an oligonucleotide (**403**) containing a 3' X' sequence and a target specific priming sequence (the 1' sequence) under hybridization/annealing conditions. The target specific sequence 1' is designed to flank one side of the region of interest (the 1' sequence adjacent to E in the genomic insert; note that only the E-containing polynucleotide fragment is shown in FIG. **4**), much like a PCR primer. After annealing primer **403**, the hybridized complex is extended, whereby all of the adaptor tagged fragments will obtain the complement of the target specific sequence (i.e., the 1 sequence) on the 3' end (see structure **405**; arrows denote the direction of extension).

Extended products **405** are then denatured and the 1/1' regions allowed to hybridize intramolecularly in a reflex process priming event, after which nucleic acid extension is performed to form structure **407** (extension is from the 1 priming site; shown with an arrow). This reflex reaction creates a product (**407**) that, unlike its parent structure (**405**), has a sequencing primer site (454A) that is oriented such the extension using this primer sequence proceeds towards the region of interest. Thus, in the absence of a priming and extension reflex reaction, extension with a sequencing primer will not generate a product containing the region of interest (the E region). In other words, only E-region containing target polynucleotides will have a 454A sequence that can amplify genomic material (structure **407**).

After completing the reflex process (using 1/1' as the reflex sequences), a PCR amplification reaction is performed to amplify the region of interest (with associated adapter domains). However, before performing the PCR reaction, the fragment sample is "inactivated" from further extension using terminal transferase and ddNTPs. This inactivation prevents non-target adaptor tagged molecules from performing primer extension from the 3' primer 1 site. Once inactivated, a PCR reaction is performed using a sequencing primer (i.e., 454A primer **409**) and a second primer that primes and extends from the opposite side of the region of interest (i.e., primer **411**, which includes a 5' 454B sequencing primer site and a 3' "2" region that primes on the opposite end of E from the 1 region). Only fragments that have undergone the reflex process and contain the E region will be suitable templates for the PCR reaction and produce the desired product (**413**).

Thus, the process exemplified in FIG. **4** allows for the movement of an adapter domain (e.g., containing functional elements and/or MID) into proximity to a desired region of interest.

The reflex process described herein can be used to perform powerful linkage analysis by combining it with nucleic acid counting methods. Any convenient method for tagging and/or counting individual nucleic acid molecules with unique tags may be employed (see, e.g., U.S. Pat. No. 7,537,897, issued on May 26, 2009; U.S. Pat. No. 7,217,522, issued on May 15, 2007; U.S. patent application Ser. No. 11/377,462, filed on Mar. 16, 2006; and U.S. patent application Ser. No. 12/432,080, filed on Apr. 29, 2009; each of which is incorporated by reference herein in their). All of this can be conducted in parallel thus saving on the cost of labor, time and materials.

In one exemplary embodiment, a large collection of sequences is tagged with MID such that each polynucleotide molecule in the sample has a unique MID. In other words, each polynucleotide in the sample (e.g., each individual double stranded or single stranded polynucleotide) is tagged with a MID that is different from every other MID on every other polynucleotide in the sample. In general, to accomplish such molecular tagging the number of distinct MID tags to be used should be many times greater than the actual number of molecules to be analyzed. This will result in the majority of individual nucleic acid molecules being labeled with a unique ID tag (see, e.g., Brenner et al., Proc. Natl. Acad. Sci. USA. 2000 97(4):1665-70). Any sequences that then result from the reflex process on that particular molecule (e.g., as described above) will thus be labeled with the same unique MID tag and thus inherently linked. Note that once all molecules in a sample are individually tagged, they can be manipulated and amplified as much as needed for processing so long as the MID tag is maintained in the products generated.

For example, we might want to sequence one thousand viral genomes (or a specific genomic region) or one thousand copies of a gene present in somatic cells. After tagging each polynucleotide in the sample with a sequencing primer site, MID and reflex sequence (as shown in the figures and described above), we use the reflex process to break each polynucleotide into lengths appropriate to the sequencing procedure being used, transferring the sequencing primer site and MID to each fragment (as described above). Obtaining sequence information from all of the reflex-processed samples can be used to determine the sequence of each individual polynucleotide in the starting sample, using the MID sequence to defining linkage relationships between sequences from different regions in the polynucleotide being sequenced. Using a sequencing platform with longer read lengths can minimize the number of primers to be used (and reflex fragments generated).

The advantages noted above are shown in FIG. **5**. This figure shows a comparison of methods for identifying nucleic acid polymorphisms in homologous nucleic acids in a sample (e.g., the same region derived from a chromosomal pair of a diploid cell or viral genomes/transcripts). The top schematic shows two nucleic acid molecules in a sample (1 and 2) having a different assortment of polymorphisms in polymorphic sites A, B and C (A1, B1, C1 and C2). Standard sequencing methods using fragmentation (left side) can identify the polymorphisms in these nucleic acids but do not retain linkage information. Employing the reflex process described herein to identify polymorphisms (right side) maintains linkage information. It is noted that not all domain structures and steps are shown in the reflex process for simplicity.

Kits and Systems

Also provided by the subject invention are kits and systems for practicing the subject methods, as described above, such vectors configured to add reflex sequences to nucleic acid inserts of interest and regents for performing any steps in the cloning or reflex process described herein (e.g., restriction enzymes, nucleotides, polymerases, primers, exonucleases, etc.). The various components of the kits may be present in separate containers or certain compatible components may be precombined into a single container, as desired.

The subject systems and kits may also include one or more other reagents for preparing or processing a nucleic acid sample according to the subject methods. The reagents may include one or more matrices, solvents, sample preparation reagents, buffers, desalting reagents, enzymatic reagents, denaturing reagents, where calibration standards such as positive and negative controls may be provided as well. As such, the kits may include one or more containers such as vials or bottles, with each container containing a separate component for carrying out a sample processing or preparing step and/or for carrying out one or more steps of a nucleic acid variant isolation assay according to the present invention.

In addition to above-mentioned components, the subject kits typically further include instructions for using the components of the kit to practice the subject methods, e.g., to prepare nucleic acid samples for perform the reflex process according to aspects of the subject methods. The instructions for practicing the subject methods are generally recorded on a suitable recording medium. For example, the instructions may be printed on a substrate, such as paper or plastic, etc. As such, the instructions may be present in the kits as a package insert, in the labeling of the container of the kit or components thereof (i.e., associated with the packaging or sub-packaging) etc. In other embodiments, the instructions are present as an electronic storage data file present on a suitable computer readable storage medium, e.g. CD-ROM, diskette, etc. In yet other embodiments, the actual instructions are not present in the kit, but means for obtaining the instructions from a remote source, e.g. via the internet, are provided. An example of this embodiment is a kit that includes a web address where the instructions can be viewed and/or from which the instructions can be downloaded. As with the instructions, this means for obtaining the instructions is recorded on a suitable substrate.

In addition to the subject database, programming and instructions, the kits may also include one or more control samples and reagents, e.g., two or more control samples for use in testing the kit.

Utility

The reflex process described herein provides significant advantages in numerous applications, a few of which are noted below (as well as described above).

For example, as described above, certain aspects of the reflex process define the particular sections of sequence to be analyzed by a primer pair, as in PCR (e.g., the two oligos shown as A_n-B_n in FIG. **1**B). This results in higher sequence specificity as compared to other extraction methods (e.g., using probes on a microarray) that only use a single oligo sequence. The separation of the probes defines a length that can be relatively uniform (hence making subsequent handling including amplification more uniform) and can also be tailored to the particular sequencing platform being employed.

Further, as described above, aspects of the present invention can be used to analyze homologous genomic locations in a multiplexed sample (i.e., a sample having polynucleotides from different genomic samples) in which the polynucleotides are tagged with the MID. This is possible because the reflex process, which operates intramolecularly, maintains the MID thus linking any particular fragment to the sample from which it originates.

Finally, as the reflex processes described herein function intramolecularly, one can determine the genetic linkage between different regions on the same large fragment that are too far apart to be sequenced in one sequence read. Such a determination of linkage may be of great value in plant or animal genetics (e.g., to decide if a particular set of variations are linked together on the same stretch of chromosome) or in viral studies (e.g., to determine if particular variations are linked together on the same stretch of a viral genome/transcripts, e.g., HIV, hepatitis virus, etc.).

EXAMPLES

Example I

FIGS. **6** and **7** provide experimental data and validation of the reflex process described herein using synthetic polynucleotide substrates.

Methods

Substrate:

The 100 base oligonucleotide substrate (as shown diagrammatically in FIG. **6**A) was synthesized with internal fluorescein-dT positioned between the REFLEX and REFLEX' sequences. This label provides convenient and sensitive method of detection of oligonucleotide species using polyacrylamide gel electrophoresis.

Extension Reactions:

Reactions were prepared which contained 1 μM of the 100 base oligonucleotide substrate, 200 μM dNTPs, presence or

absence of 1 μM competitor oligonucleotide, 0.5 μl of each DNA polymerase ("DNAP"): Vent (NEB, 2 units/μl), Taq (Qiagen HotStarTaq 5 units/μl) and Herculase (Stratagene), and made up to 50 μl with the appropriate commercial buffers for each polymerase and dH_2O. For Taq titrations 0.54 μl, 1 μl, 2 μl, and 3 μl enzyme was used in the same 50 μl volume. Reactions were heated in a Biometra thermocycler to 95° C. for 15 minutes (Taq) or 5 minutes (Herculase, Vent), followed by 55° C. or 50° C. for 30 seconds, and a final incubation at 72° C. for 10 minutes.

T7 Exonuclease Digestions:

Reactions were prepared with 10 μl extension reactions above, 0.5 μl T7 exonuclease (NEB, 10 units/μl), and made up to 50 μl using NEB Buffer 4 and dH_2O. Reactions were incubated at 25° C. for 30 minutes.

Gel Electrophoresis Analysis:

An 8% denaturing polyacrylamide gel was used to analyze reaction species. 0.4 μl of extension reactions, and 2 μl of digestion reactions were loaded and ran at 800V for ~1.5 hours. Gels were analyzed for fluorescein using an Amersham/General Electric Typhoon imager.

Results

FIG. **6**A shows the structure of each stage of reflex sequence processing with the expected nucleic acid size shown on the left. The initial single stranded nucleic acid having a sequencing primer site (the Roche 454 sequencing primer A site; listed as 454A); an MID; a reflex sequence; the insert; and a complement of the reflex sequence is 100 nucleotides in length. After self-annealing and extension, the product is expected to be 130 nucleotides in length. After removal of the double stranded region from the 5' end, the nucleic acid is expected to be 82 bases in length.

FIG. **6**B shows the results of three experiments using three different nucleic acid polymerases (Vent, Herculase and Taq, indicated at the top of the lanes). The temperature at which the annealing was carried out is shown at the top of each lane (either 50° C. or 55° C.). The sizes of the three nucleic acids as noted above are indicated on the left and right side of the gel.

As shown in FIG. **6**B, extension appears to be most efficient under the conditions used with Herculase (Herculase is a mixture of two enzymes: modified Pfu DNAP and Archaemax (dUTPase)). Most (or all) of the initial 100 base pair nucleic acid are converted to the 130 base pair product (see lanes 6 and 7). However, after T7 exonuclease digestion the 3'-5' exonuclease activity of Herculase results in partial digestion of the desired 82 base product (note bands at and below the 82 base pairs in lanes 8 and 9).

Taq, which lacks 3'-5' exonuclease activity, shows a stronger band at the expected size of the final product after T7 exonuclease digestion (see lane 13).

FIG. **7** shows the effect on the reflex process of increasing amounts of Taq polymerase as well as the use of a reflex sequence competitor (schematically shown in FIG. **7**A).

As shown in lanes 2 to 5, increased Taq concentration improves extension to ~90% conversion of the starting nucleic acid (see lane 5). Lanes 7 to 8 show that T7 exonuclease digestion does not leave a perfect 82 base product. This may be due to collapse of dsDNA when T7 exonuclease has nearly completed its digestion from the 5' end in the double stranded region of the fold-back structure. It is noted that in many embodiments, the removal of a few additional bases from the 5' end of the polynucleotide will not interfere with subsequent analyses, as nucleotide bases at the 5' end are often removed during subsequent steps.

As shown in Lanes 11-14, addition of a competitor (which can interfere with annealing of the reflex sequences to form the fold-back structure) results in only a small decrease (~5-10%) of fully extended product. Thus, as expected, the intramolecular reaction is heavily favored. Although not shown, we have observed that the competitor oligonucleotide also gets extended by the same amount (~5-10%).

The concentration of the competitor, the concentration of the reflex substrate, and the overall genetic complexity, will all likely affect specific results. The experiments shown in FIGS. **6** and **7** demonstrate that the core parts of the reflex processes as described herein is functional and can be implemented.

Example II

FIG. **8** shows the reflex workflow (diagram at left) and exemplary results of the workflow (gel at right) for a specific region of interest (ROI). The starting material is a double stranded nucleic acid molecule (**700**) that contains a 454A primer site, an MID, a reflex site, and a polynucleotide of interest having three ROIs (2, 3 and 4) at different locations therein. This starting material was subjected to reflex processes (as described in above) specific for ROI 2 as shown in the diagram at the left of the figure, both with and without the use of a T7 exonuclease step (the T7 exonuclease step is shown in the diagram is indicated as "Optional").

Completion of all steps shown in the reflex process should result in a double stranded polynucleotide of 488 base pairs (**702**) with or without the T7 exonuclease step.

As shown in the gel on the right of FIG. **8**, the 488 base pair product was produced in reflex processes with and without the T7 exonuclease step.

FIG. **9** shows an exemplary protocol for a reflex process based on the results discussed above. The diagram shows specific reflex process steps with indications on the right as to where purification of reaction products is employed (e.g., using Agencourt SPRI beads to remove primer oligos). One reason for performing such purification steps is to reduce the potential for generating side products in a reaction (e.g., undesirable amplicons). While FIG. **9** indicates three purification steps, fewer or additional purification steps may be employed depending on the desires of the user. It is noted that the steps of reversing polarity, reflex priming and extension, and "stretch out" (or denaturation)/second reversing polarity step can be performed without intervening purification steps.

The protocol shown in FIG. **9** includes the following steps:

annealing a first primer containing a 5' reflex sequence (or reflex tail, as noted in the figure) specific for the 3' primer site for the R' region to the starting polynucleotide and extending (the primer anneals to the top strand at the primer site at the right of R in polynucleotide **902**, indicated with a *; this step represents the first denature, anneal and extend process indicated on the right);

after purification, adding a 454A primer and performing three cycles of denaturing, annealing and extending: the first cycle results in the copy-back from the 454A primer to reverse the polarity of the strand just synthesized; the second cycle breaks apart the double stranded structure produced, allows the reflex structure to form and then extend; the third cycle results in another copy-back using the same 454A primer originally added; after purification, adding a second primer specific for the second primer site for the R' region having a 5' 454B tail (this primer anneals to the primer site 3' of the R' region in polynucleotide **904**, indicated with a *) and denaturing, annealing and extending resulting in a polynucleotide product having 454A and 454B sites sur-

rounding the MID, the reflex sequence, and R'. Note that the first primer specific for the R' region and the second primer specific for the R' region define its boundaries, as described above and depicted in FIG. **1**B);

after another purification, adding 454A and 454B primers and performing a PCR amplification reaction.

Example III

As described above, a reflex sequence can be an "artificial" sequence added to a polynucleotide as part of an adapter or can be based on a sequence present in the polynucleotide of interest being analyzed, e.g., a genomic sequence (or "non-artificial").

The data shown in prior Examples used "artificial" reflex sites. In this Example, the reflex site is a genomic sequence present in the polynucleotide being analyzed.

The starting material is a double stranded DNA containing a 454A site, an MID and a polynucleotide to be analyzed. The 454A and MID were added by adapter ligation to parent polynucleotide fragments followed by enrichment of the polynucleotide to be analyzed by a hybridization-based pull-out reaction and subsequent secondary PCR amplification (see Route 1 in FIG. **13**). Thus, the reflex site employed in this example is a sequence normally present at the 5' end of the subject polynucleotide (a genomic sequence). The polynucleotide being analyzed includes a region of interest distal to the 454A and MID sequences that is 354 base pairs in length.

This starting double stranded nucleic acid is 755 base pairs in length. Based on the length of each of the relevant domains in this starting nucleic acid, the reflex process should result in a product of 461 base pairs.

FIG. **10** shows the starting material for the reflex process (left panel) and the resultant product generated using the reflex process (right panel; reflex process was performed as described in Example II, without using a T7 exonuclease step). A size ladder is included in the left hand lane of each gel to allow estimation of the size of the test material. This figure shows that the 755 base pair starting nucleic acid was processed to the expected 461 base pair product, thus confirming that a "non-artificial" reflex site is effective in moving an adapter domain from one location to another in a polynucleotide of interest in a sequence specific manner.

Example IV

FIG. **11** shows a schematic of an experiment in which the reflex process is performed on a single large initial template (a "parent" fragment) to generate 5 different products ("daughter" products) each having a different region of interest (i.e., daughter products are produced having either region 1, 2, 3, 4 or 5). The schematic in FIG. **11** shows the starting fragment (11,060 base pairs) and resulting products (each 488 base pairs) generated from each of the different region of interest-specific reflex reactions (reflex reactions are performed as described above). The panel (gel) on the bottom of FIG. **11** shows the larger starting fragment (Lane 1) and the resulting daughter products for each region-specific reflex reaction (lanes 2 to 6, with the region of interest noted in each in the box), where the starting and daughter fragments have the expected lengths. Sequencing of the products confirmed the identity of the region of interest in each of the reflex products shown in the gel. These results demonstrate that multiple different reflex products can be generated from a single, asymmetrically tagged parent fragment while maintaining the adapter domains (e.g., the primer sites and MID).

Example V

FIG. **12** details experiments performed to determine the prevalence of intramolecular rearrangement (as desired in the reflex process) vs. intermolecular rearrangement. Intermolecular rearrangement is undesirable because it can lead to the transfer of an MID from one fragment to another (also called MID switching). MID switching can occur if a reflex sequence in a first fragment hybridizes to its complement in a second fragment during the reflex process, leading to appending the MID from the second fragment to the first fragment. Thus, intermolecular rearrangement, or MID switching, should be minimized to prevent the transfer of an MID from one fragment in the sample to another, which could lead to a misrepresentation of the source of a fragment.

To measure the prevalence of MID switching under different reflex conditions, fragments having different sizes were generated that included two different MIDs, as shown in the top panel of FIG. **12**. The common sequence on these fragments serves as the priming site for the first extension reaction to add the second reflex sequence (see, e.g., step **2** of FIG. **3**). Three exemplary fragments are shown in FIG. **12** for each different fragment size (i.e., 800 base pairs with an MIDB and MIDA combination; 1900 base pairs with MIDC and MIDA combination; and 3000 base pairs with MIDD and MIDA combination). For each MID family (A, B, C and D), there are 10 different members (i.e., MIDA had 10 different members, MIDB has 10 different members, etc.). A set of 10 dual MID fragments for each different size fragment (i.e., 800, 1900 and 3000 base pairs) were generated, where the MID pairs (i.e., MIDA/MIDB, MIDA/MIDC, and MIDA/MIDD) were designated as 1/1, 2/2, 3/3, 4/4, 5/5, 6/6, 7/7, 8/8, 9/9, and 10/10. All 10 fragments of the same size were then mixed together and a reflex protocol was performed.

Due to the domain structure of the fragments, a successful reflex process results in the two MIDs for each fragment being moved to within close enough proximity to be sequenced in a single read using the Roche 454 sequencing platform (see the reflex products shown in the schematic of FIG. **12**). The reflex reactions for each fragment size were performed at four different fragment concentrations to determine the effect of this parameter, as well as fragment length, in the prevalence of MID switching. The reflex products from each reaction performed were subjected to 454 sequencing to determine the identity of both MIDs on each fragment, and thereby the proportion of MID switching that occurred.

The panel on the bottom left of FIG. **12** shows the rate of MID switching (Y axis, shown in % incorrect (or switched) MID pair) for each different length fragment at each different concentration (X axis; 300, 30, 3 and 0.3 nM). As shown in this panel, the MID switch rate decreases with lower concentrations, as would be expected, because intermolecular, as opposed to intramolecular, binding events are concentration dependent (i.e., lower concentrations lead to reduced intermolecular hybridization/binding). In addition, the MID switch rate decreases slightly with length. This is somewhat unexpected as the ends of longer DNA fragments are effectively at a lower concentration with respect to one another. The reasons for why we do not see this is probably because the production of reflex priming intermediates continues during the final PCR, which means that reflex priming

reactions are happening continuously which contributes to MID switching. It is probably the case that the shorter reflex products are able to undergo a higher rate of 'background' reflexing, and therefore increase the overall MID switch rate a little.

These results demonstrate that MID switching can be minimized (e.g., to below 2%, below 1% or even to nearly undetectable levels) by altering certain parameters of the reaction, e.g., by reducing fragment concentration and/or fragment length.

The panel on the bottom right of FIG. **12** shows the frequency of MID switching in the reflex process for the 800 base pair fragments (i.e., MIDA/MIDB containing fragments). In this figure, the area of each circle is proportional to the number of reads containing the corresponding MIDA and MIDB species (e.g., MIDA1/MIDB1; MIDA1/MIDB2; etc.). Thus, a circle representing 200 reads will be 40 times larger in terms of area than a circle representing 5 reads.

As noted above, the MIDA/MIDB combinations having the same number (shown on the X and Y axis, respectively) represent the MIDA/MIDB combinations present in the sample prior to the reflex process being performed (i.e., MIDA/MIDB combinations 1/1, 2/2, 3/3, 4/4, 5/5, 6/6, 7/7, 8/8, 9/9, and 10/10 were present in the starting sample). All other MIDA/MIDB combinations identified by Roche 454 sequencing were the result of MID switching.

This figure shows that the MID switching that occurs during the reflex process is random, i.e., that MID switching is not skewed based on the identity of the MIDs in the reaction).

Exemplary Reflex Protocols

FIG. **13** shows a diagram of exemplary protocols for performing the reflex process on pools of nucleic acids, for example, pools of nucleic acids from different individuals, each of which are labeled with a unique MID. In Route 3, a pooled and tagged extended library is subjected directly to a reflex process. In Route 2, the pooled library is enriched by target-specific hybridization followed by performing the reflex process. In Route 1 employs enrichment by PCR amplification. As shown in FIG. **13**, PCR enrichment can be performed directly on the pooled tagged extended library or in a secondary PCR reaction after a hybridization-based enrichment step has been performed (as in Route 2) to generate an amplicon substrate that is suitable for the reflex process. Additional routes for preparing a polynucleotide sample for performing a reflex process can be implemented (e.g., having additional amplification, purification, and/or enrichment steps), which will generally be dependent on the desires of the user.

Although the foregoing invention has been described in some detail by way of illustration and example for purposes of clarity of understanding, it is readily apparent to those of ordinary skill in the art in light of the teachings of this invention that certain changes and modifications may be made thereto without departing from the spirit or scope of the appended claims.

Accordingly, the preceding merely illustrates the principles of the invention. It will be appreciated that those skilled in the art will be able to devise various arrangements which, although not explicitly described or shown herein, embody the principles of the invention and are included within its spirit and scope. Furthermore, all examples and conditional language recited herein are principally intended to aid the reader in understanding the principles of the invention and the concepts contributed by the inventors to furthering the art, and are to be construed as being without limitation to such specifically recited examples and conditions. Moreover, all statements herein reciting principles, aspects, and embodiments of the invention as well as specific examples thereof, are intended to encompass both structural and functional equivalents thereof. Additionally, it is intended that such equivalents include both currently known equivalents and equivalents developed in the future, i.e., any elements developed that perform the same function, regardless of structure. The scope of the present invention, therefore, is not intended to be limited to the exemplary embodiments shown and described herein. Rather, the scope and spirit of present invention is embodied by the appended claims.

What is claimed:

1. A method of counting nucleic acids in a sample, the method comprising:

(a) providing a sample comprising a plurality of cells, wherein a cell of the plurality of cells comprises a plurality of sample polynucleotides;

(b) generating a plurality of tagged polynucleotides from the plurality of sample polynucleotides of said cell and a plurality of oligonucleotide tags, wherein a tagged polynucleotide of the plurality of tagged polynucleotides comprises:

(i) a sample sequence from a sample polynucleotide of the plurality of sample polynucleotides;

(ii) a first tag sequence distinguishing said sample polynucleotide from sample polynucleotides from other cells; and

(iii) a second tag sequence distinguishing said sample polynucleotide from other sample polynucleotides from said cell;

(c) sequencing the tagged polynucleotide to determine the sample sequence, the first tag sequence, and the second tag sequence; and

(d) using the first tag sequence and the second tag sequence to count a number of sample polynucleotides in said plurality of sample polynucleotides of said cell.

2. The method of claim **1**, wherein the method further comprises amplifying the plurality of tagged polynucleotides prior to the sequencing step (c).

3. The method of claim **1**, wherein the plurality of sample polynucleotides is selected from DNA and RNA.

4. The method of claim **1**, wherein the plurality of sample polynucleotides comprises mRNA.

5. The method of claim **1**, wherein the plurality of tagged polynucleotides is generated through at least one ligation reaction.

6. The method of claim **1**, wherein an oligonucleotide tag of said plurality of oligonucleotide tags comprises said first tag sequence and said second tag sequence.

7. The method of claim **1**, wherein an oligonucleotide tag of said plurality of oligonucleotide tags comprises a sequence that is configured to hybridize to said sample polynucleotide.

8. The method of claim **1**, wherein the plurality of tagged polynucleotides is generated by (i) hybridizing an oligonucleotide tag of said plurality of oligonucleotide tags to said sample polynucleotide and (ii) extending said oligonucleotide tag or said sample polynucleotide or both.

9. The method of claim **1**, wherein the plurality of tagged polynucleotides is generated through at least one linear amplification reaction.

10. The method of claim **1**, wherein the plurality of tagged polynucleotides is generated through at least one reverse transcription reaction.

11. The method of claim **1**, wherein the plurality of tagged polynucleotides is generated through at least one polymerase chain reaction (PCR).

12. The method of claim **1**, wherein substantially every sample polynucleotide of said plurality of sample polynucleotides is associated with the same first tag sequence.

13. The method of claim **1**, wherein at least 90 percent of said plurality of sample polynucleotides is associated with a unique second tag sequence.

14. The method of claim **1**, wherein at least 95 percent of said plurality of sample polynucleotides is associated with a unique second tag sequence.

15. The method of claim **1**, wherein at least 99 percent of said plurality of sample polynucleotides is associated with a unique second tag sequence.

16. The method of claim **1**, wherein substantially every sample polynucleotide of said plurality of sample polynucleotides is associated with a unique second tag sequence.

17. The method of claim **1**, wherein the number of different second tag sequences is larger than the number of sample polynucleotides.

18. The method of claim **17**, wherein the number of different second tag sequences is at least ten times the number of sample polynucleotides.

19. The method of claim **18**, wherein the number of different second tag sequences is at least one hundred times the number of sample polynucleotides.

20. The method of claim **1**, wherein said plurality of sample polynucleotides are randomly associated with said plurality of oligonucleotide tags to generate said plurality of tagged polynucleotides.

21. The method of claim **1**, wherein step (c) comprises hybridizing said tagged polynucleotide to a solid support.

22. The method of claim **20**, wherein said solid support is a bead.

23. The method of claim **1**, wherein step (d) comprises using said first tag sequence to distinguish (i) the number of sample polynucleotides having said sample sequence from said cell from (ii) the number of sample polynucleotides having said sample sequence from other cells.

24. The method of claim **1**, wherein step (d) comprises determining the number of different second tag sequences associated with said sample sequence, thereby estimating the number of sample polynucleotides having said sample sequence from said cell.

25. The method of claim **1**, wherein said plurality of sample polynucleotides comprises substantially all mRNA molecules of said cell.

26. The method of claim **1**, wherein said plurality of sample polynucleotides comprises a subset of polynucleotides of said cell having the same sequence.

* * * * *

Exhibit C

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(12) **United States Patent**
Hindson et al.

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(54) **METHODS AND SYSTEMS FOR PROCESSING POLYNUCLEOTIDES**

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This patent is subject to a terminal disclaimer.

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(60) Provisional application No. 61/737,374, filed on Dec. 14, 2012, provisional application No. 61/844,804, filed on Jul. 10, 2013, provisional application No. 61/840,403, filed on Jun. 27, 2013, provisional application No. 61/800,223, filed on Mar. 15, 2013, provisional application No. 61/762,435, filed on Feb. 8, 2013, provisional application No. 61/683,192, filed on Aug. 14, 2012.

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(58) **Field of Classification Search**

CPC C12Q 1/6806; C12Q 1/6869; C12Q 2563/149; C12Q 2563/179

See application file for complete search history.

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(57) **ABSTRACT**

The present disclosure provides compositions, methods, systems, and devices for polynucleotide processing. Such polynucleotide processing may be useful for a variety of applications, including polynucleotide sequencing.

74 Claims, 22 Drawing Sheets

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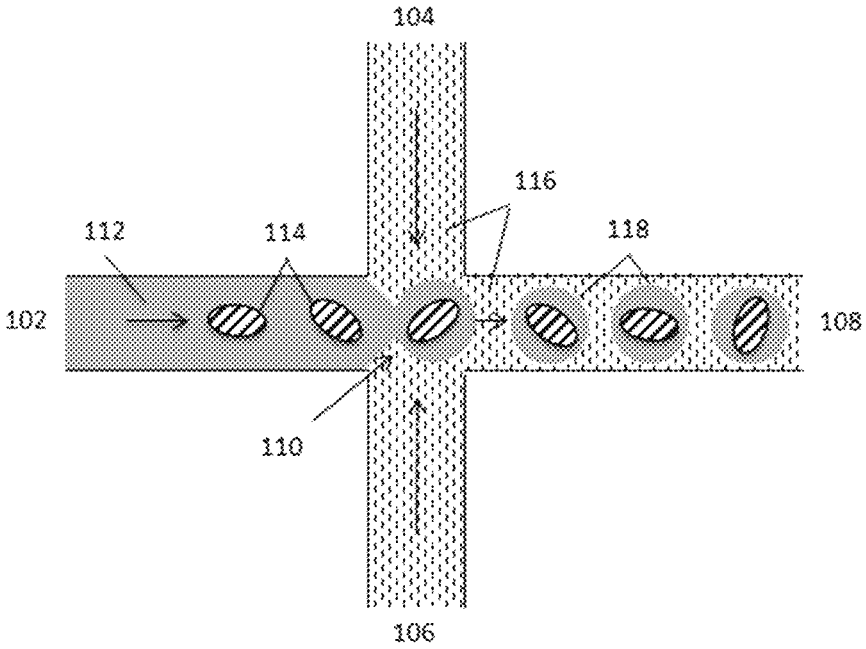

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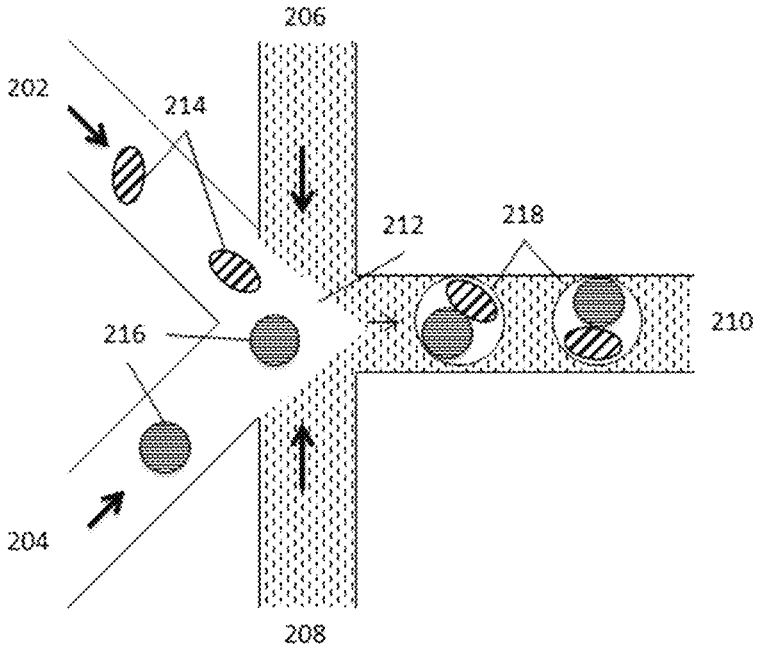

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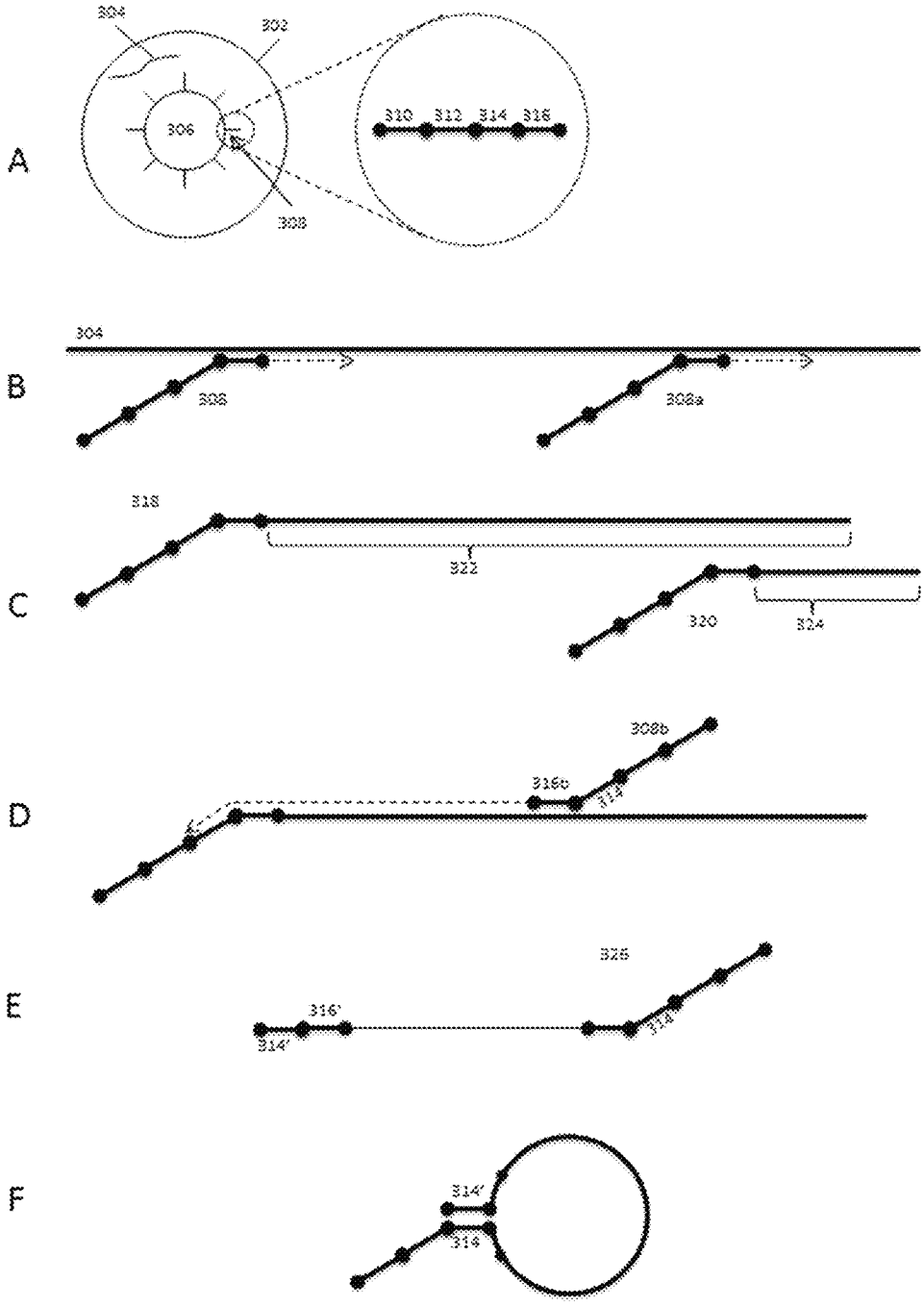
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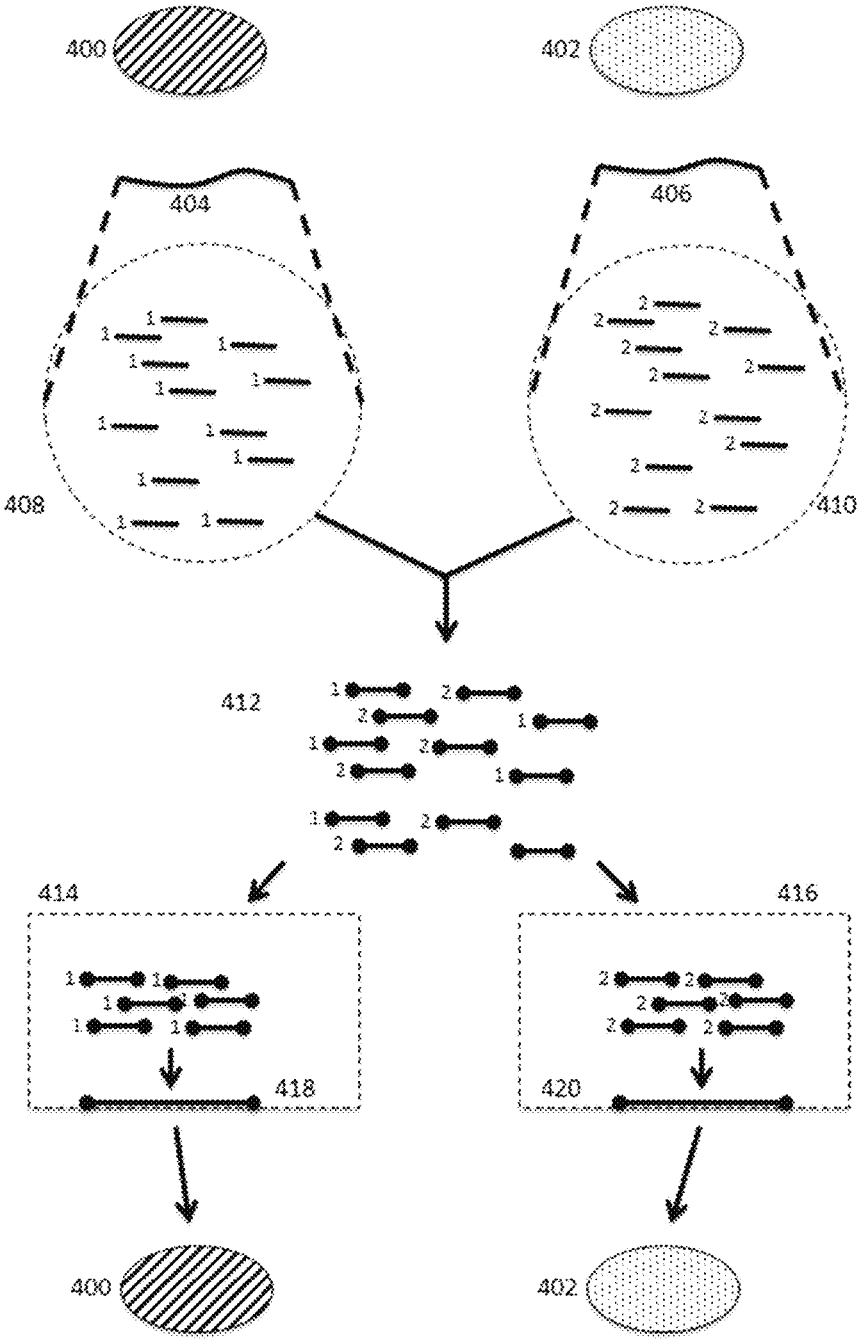
Figure 3



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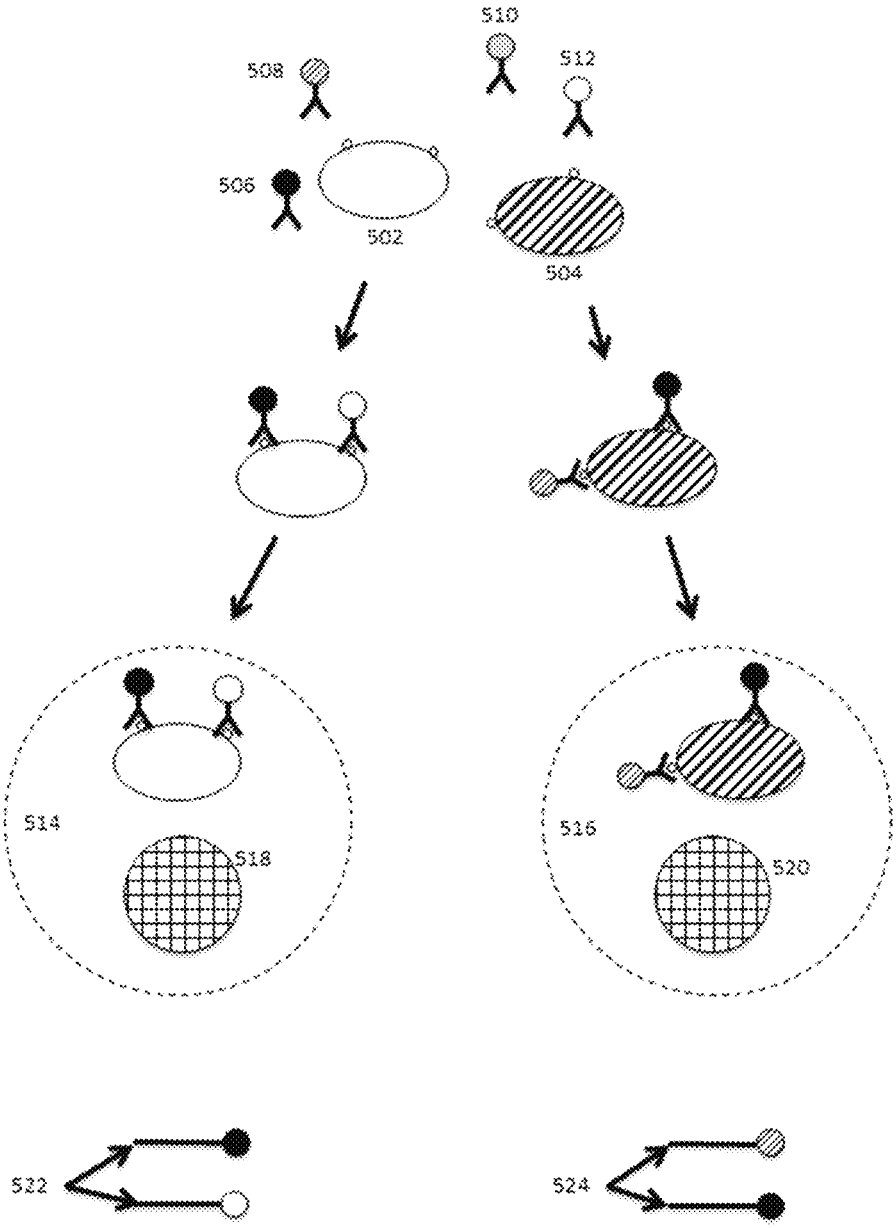

Figure 5

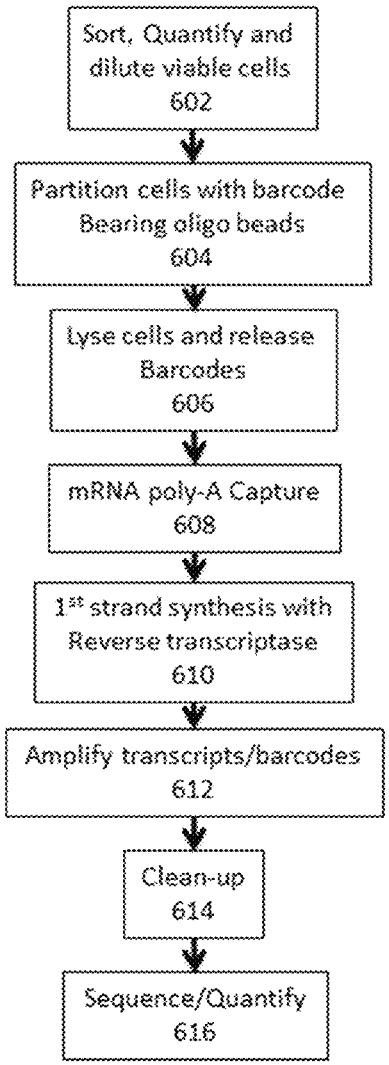
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Figure 6

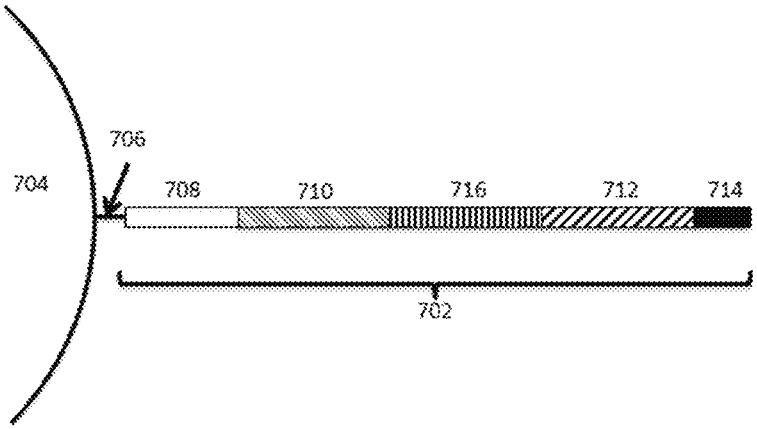

Figure 7

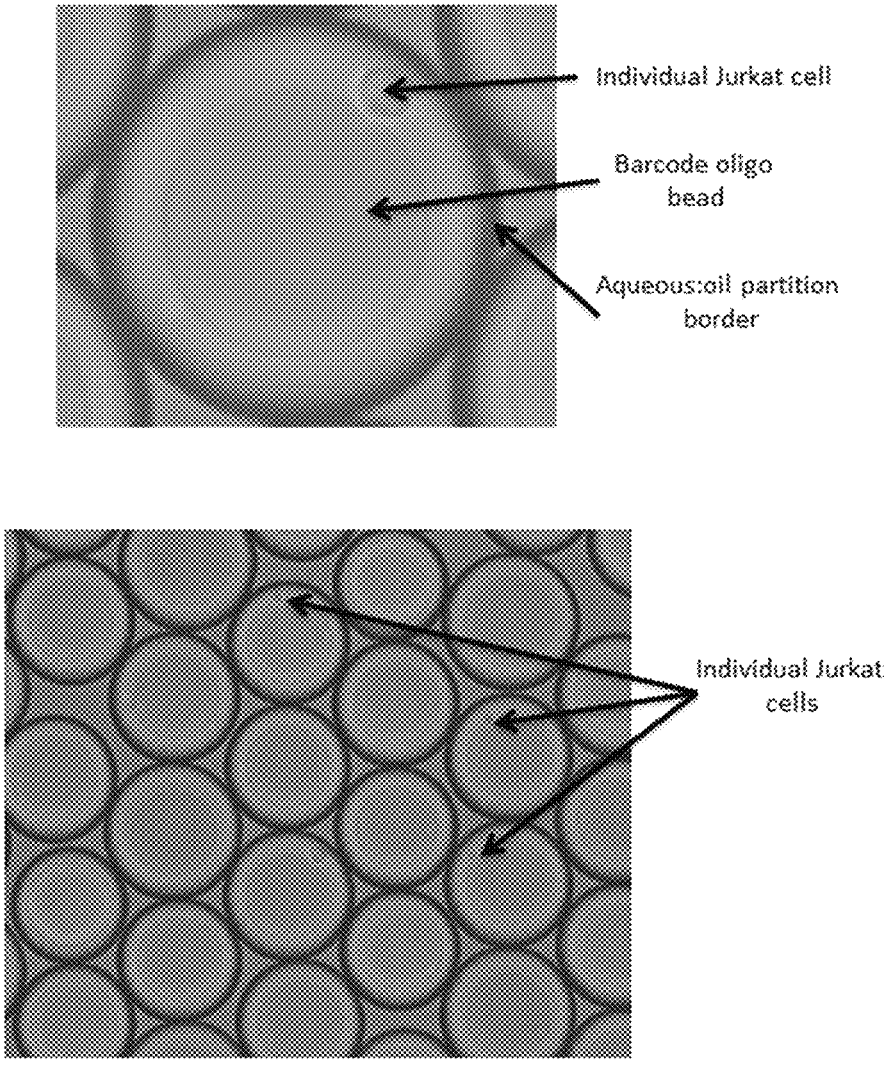
Individual Jurkat cell
Barcode oligo bead
Aqueous:oil partition border
Individual Jurkat cells

Figure 8

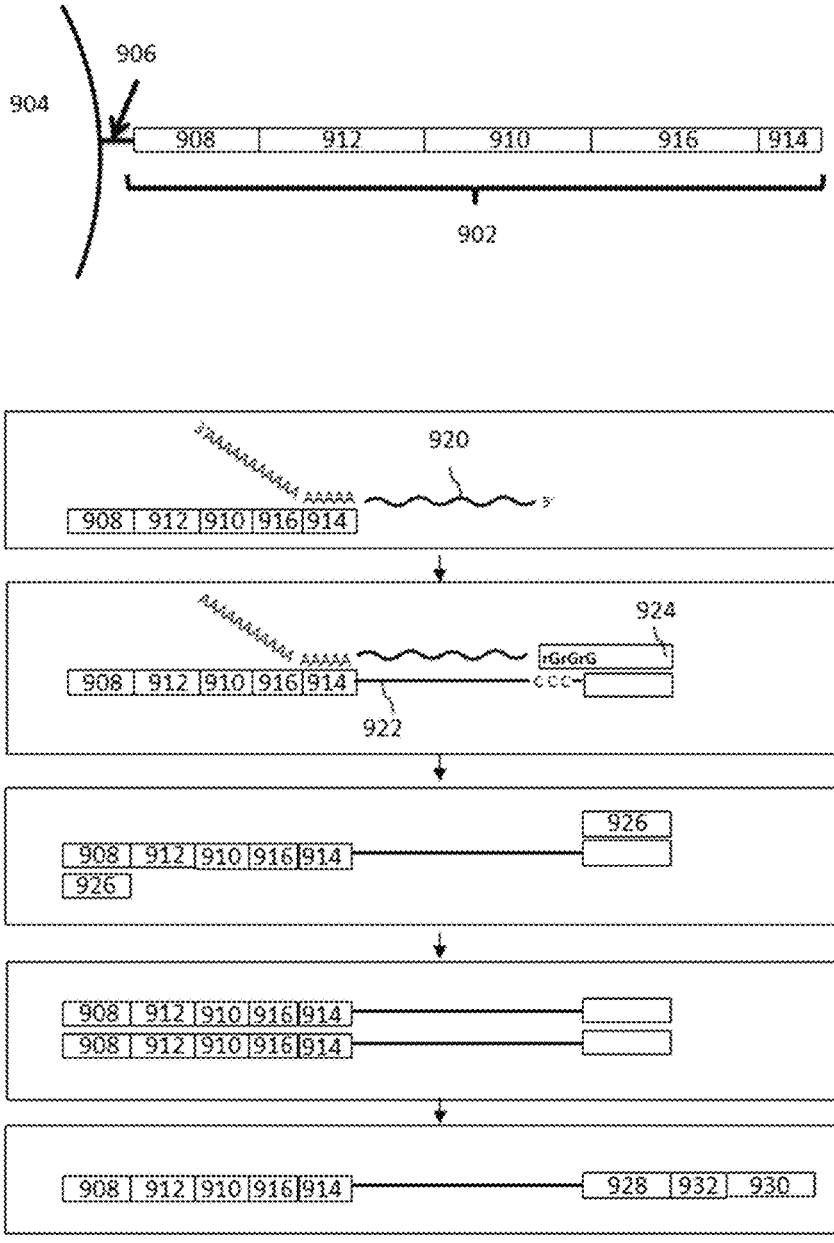
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Figure 9A

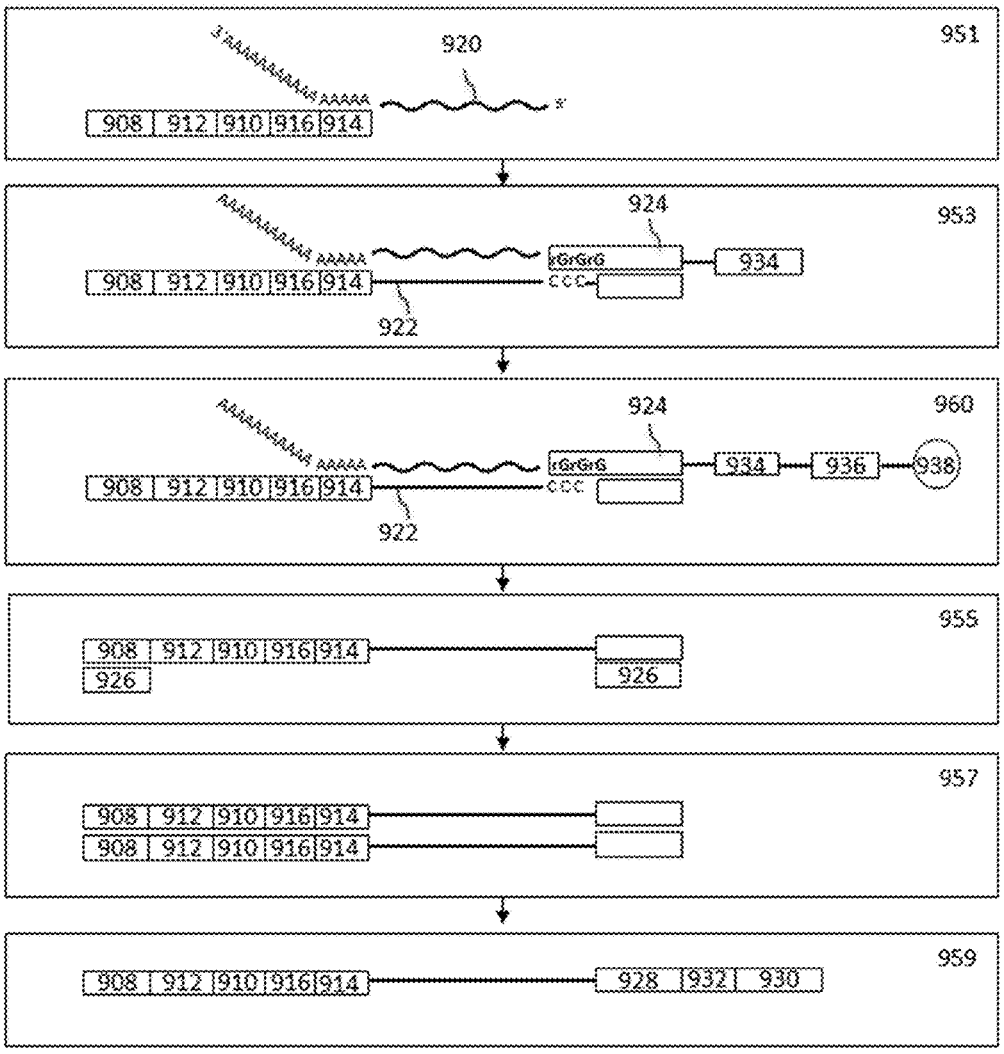
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Figure 9B

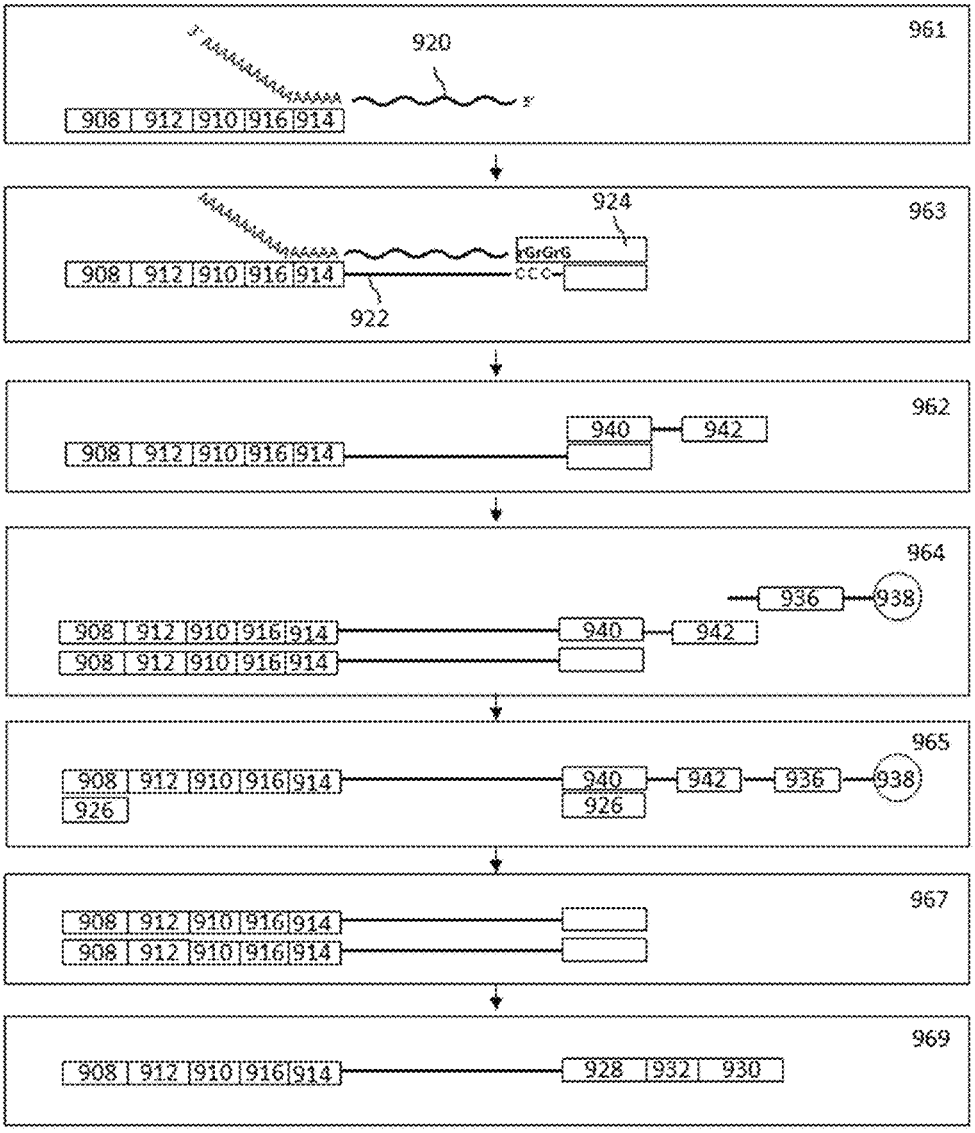
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Figure 9C

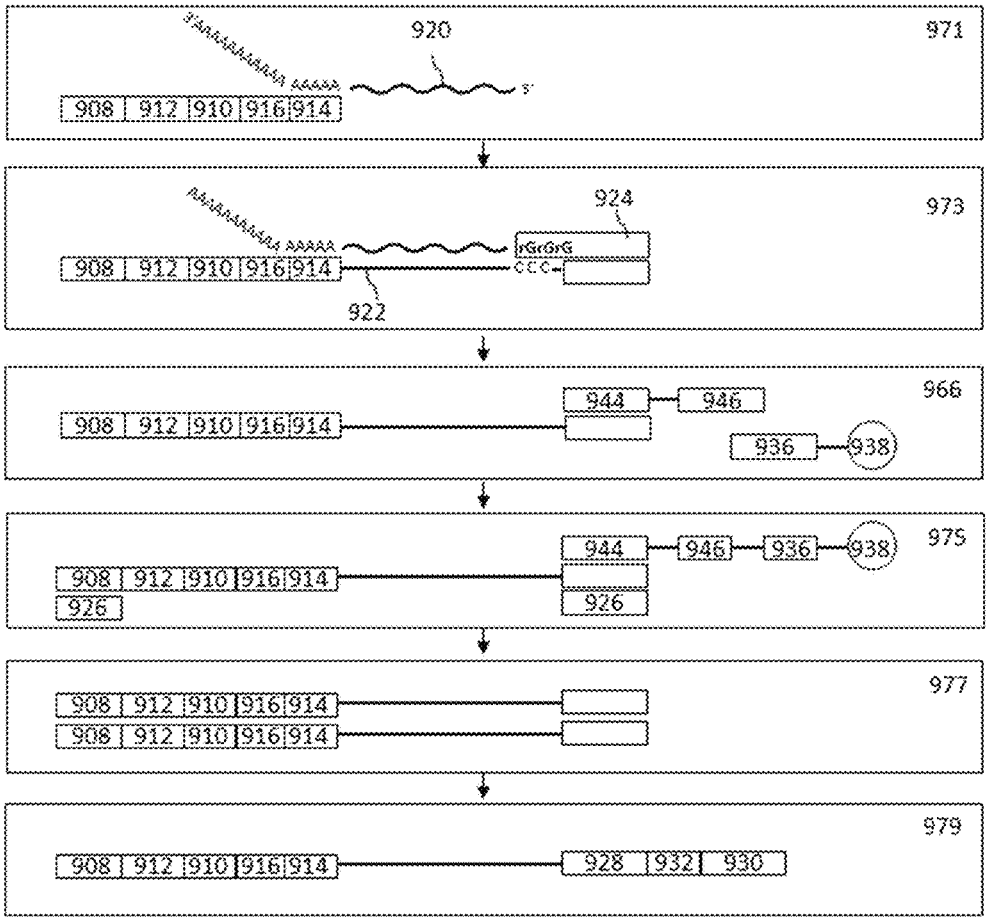
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Figure 9D

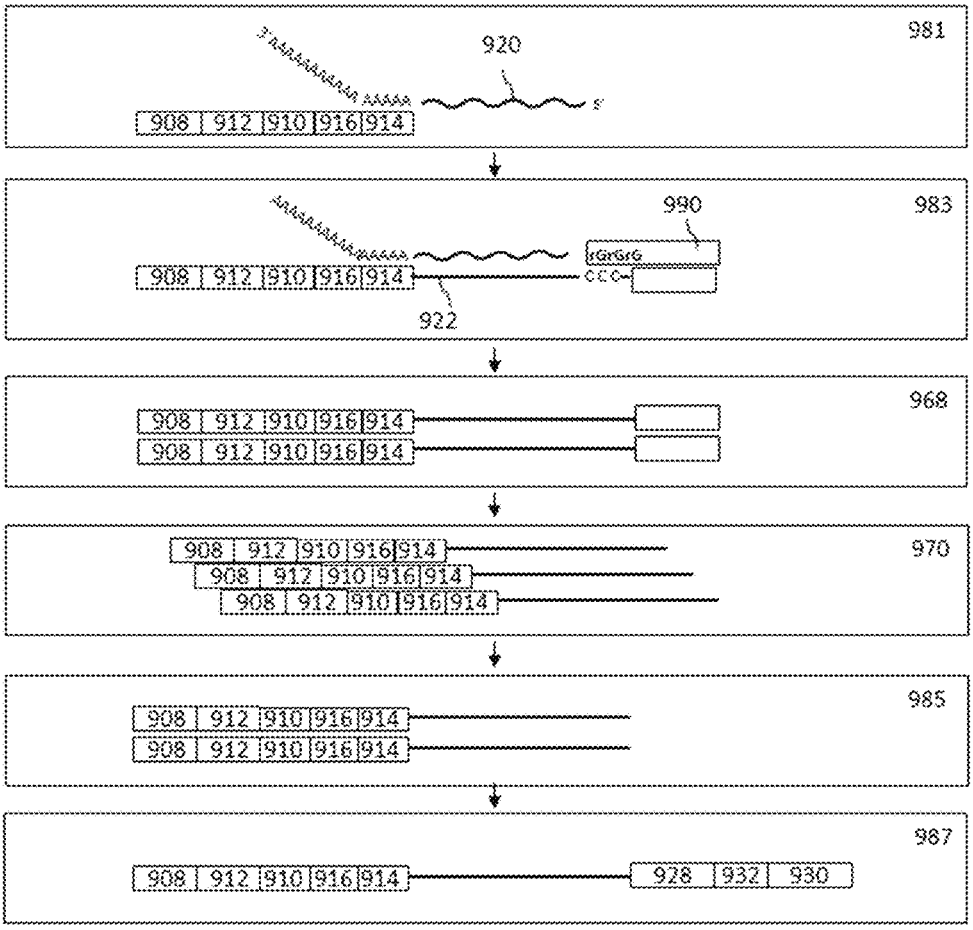
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Figure 9E

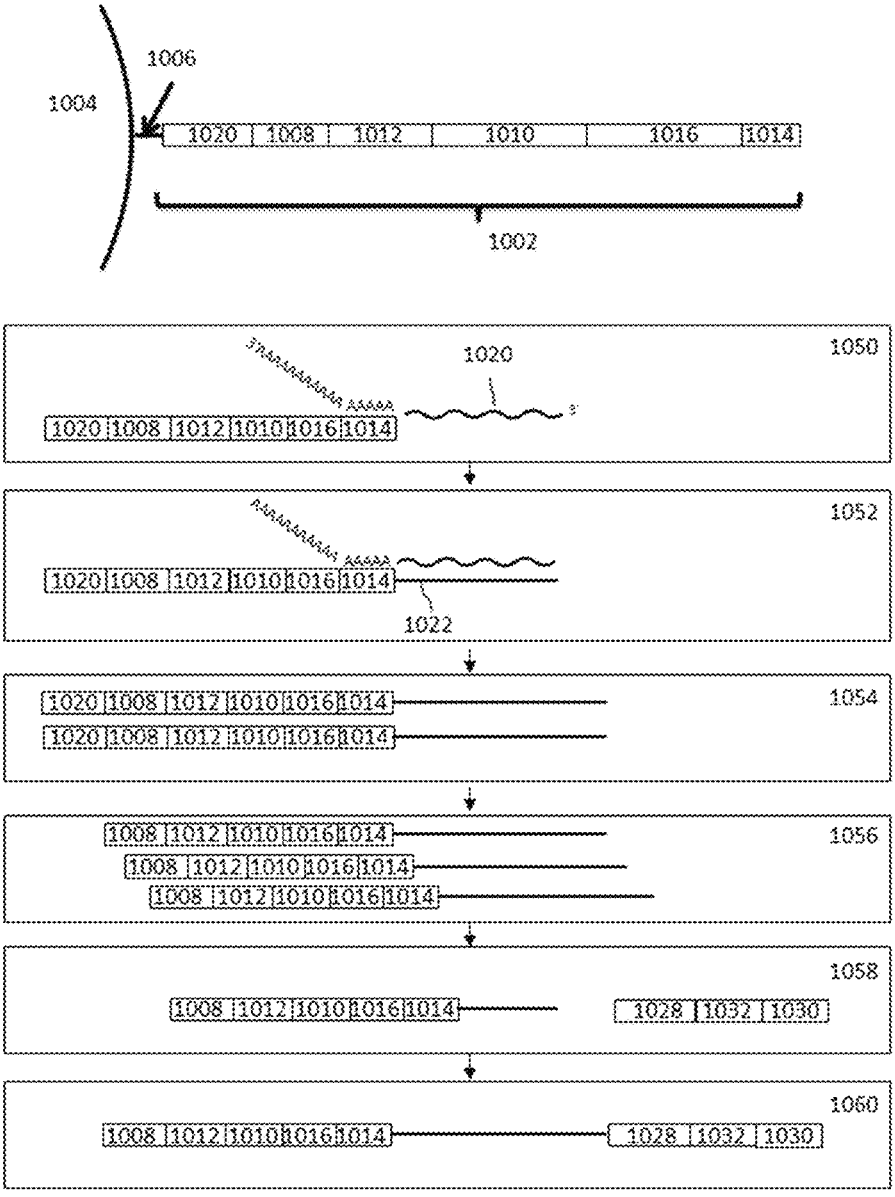
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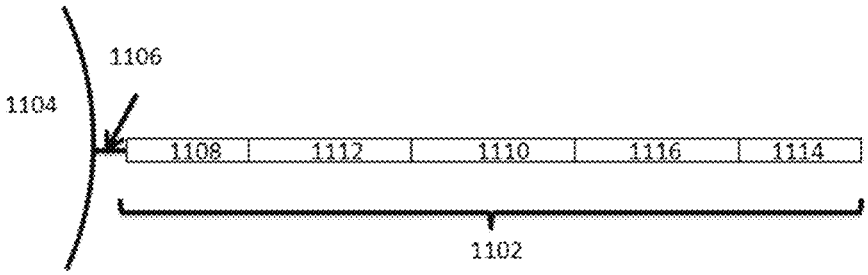
Figure 10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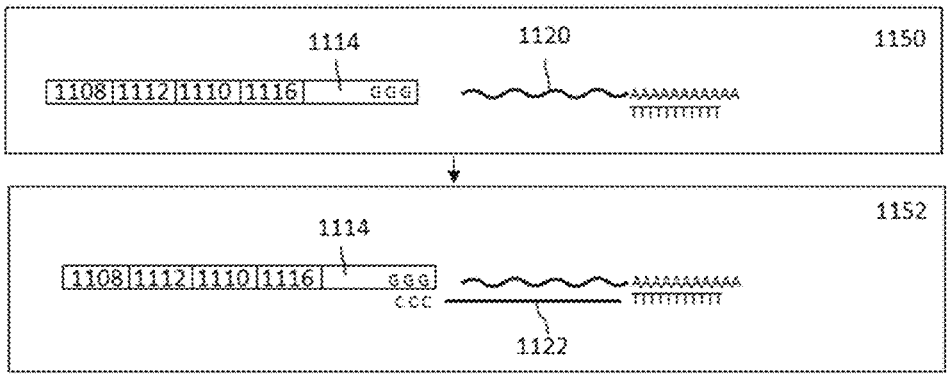
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Figure 11

Figure 12A

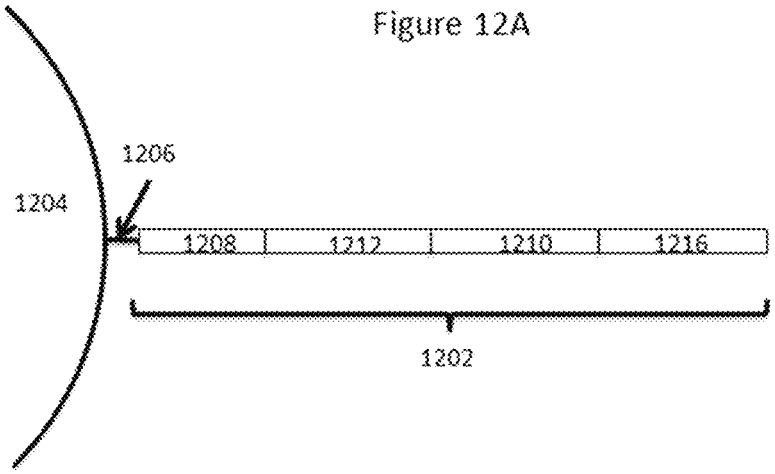


Figure 12B

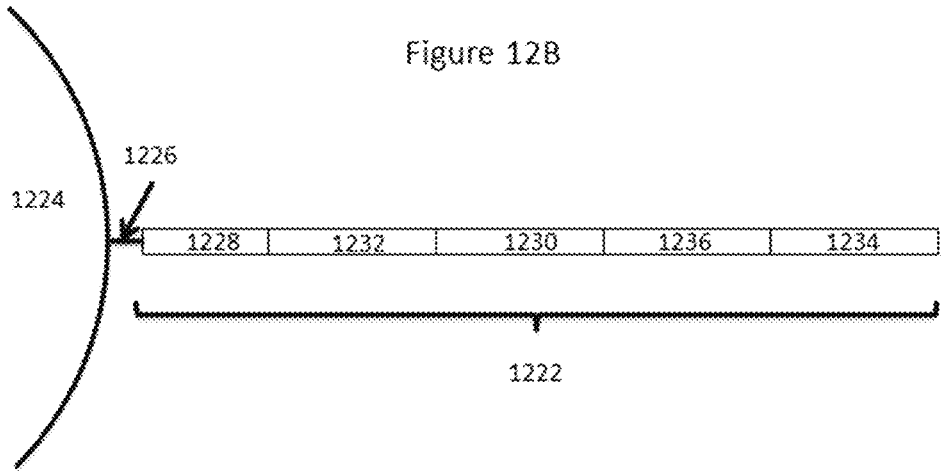


Figure 13A

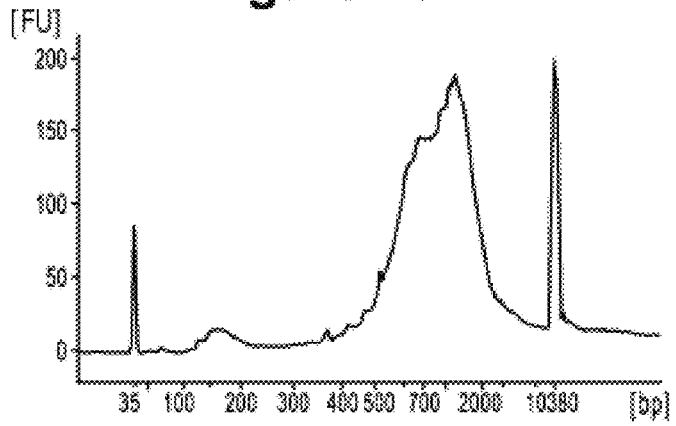


Figure 13B

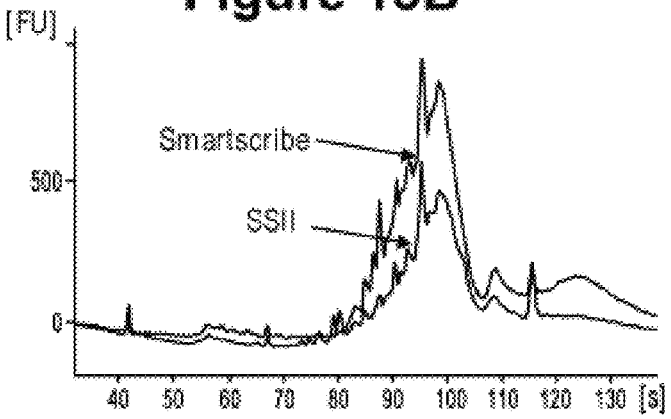


Figure 13C

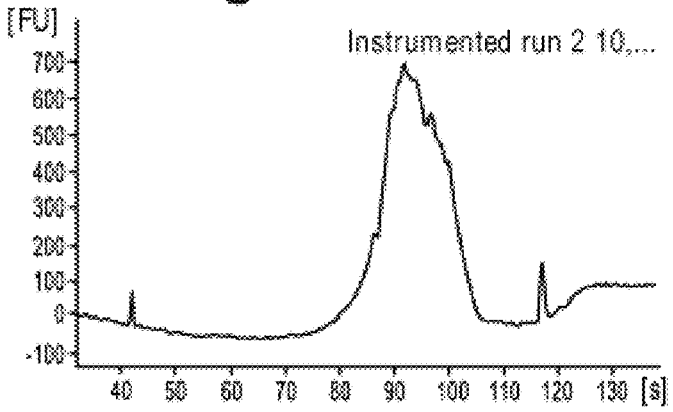
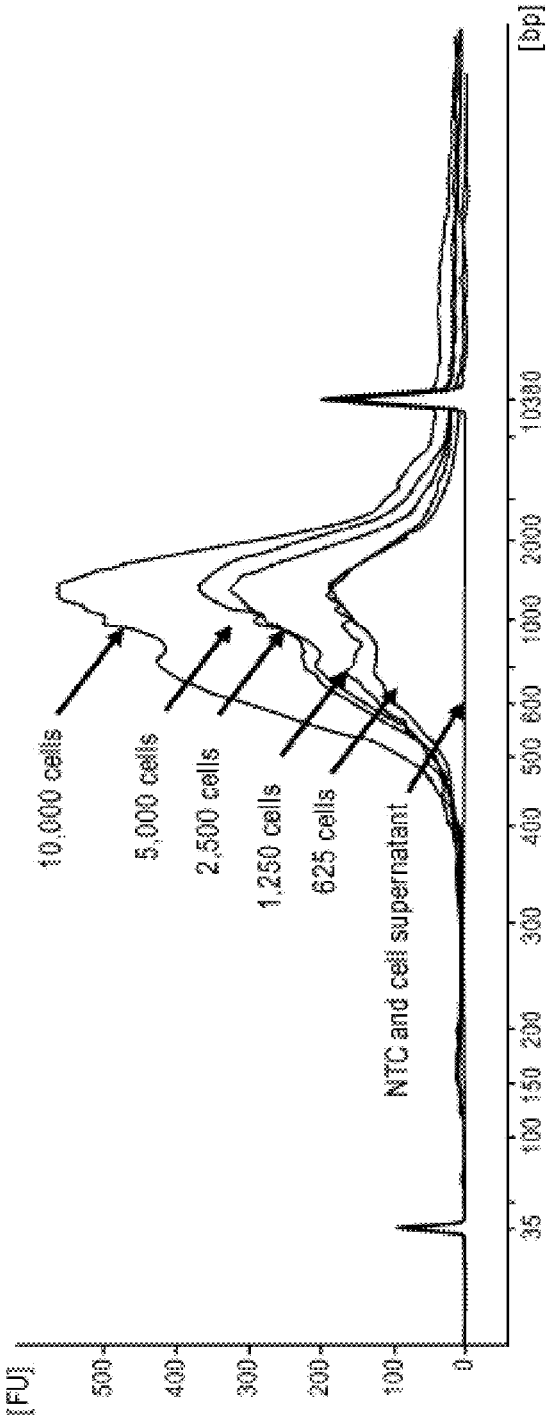


Figure 14A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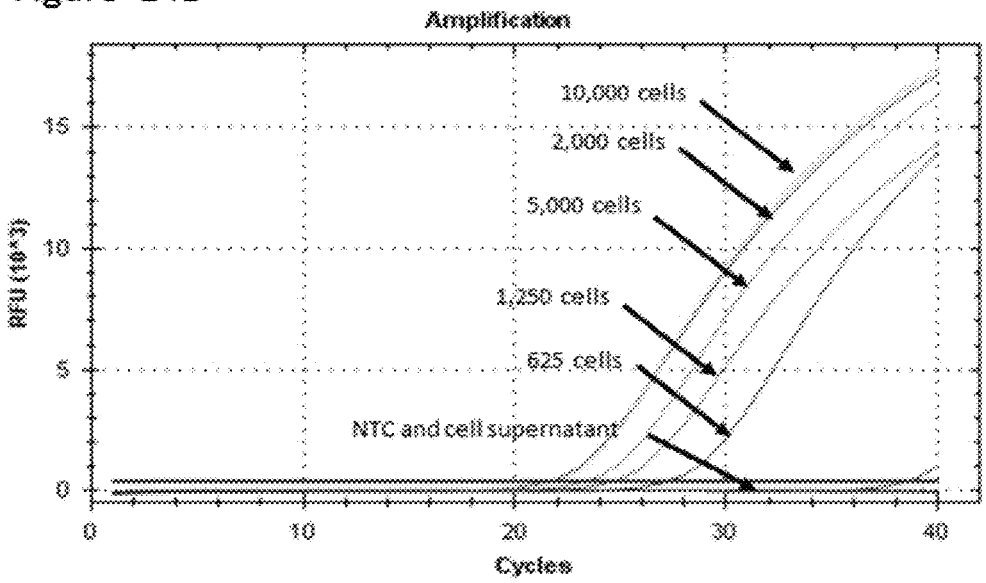
400
300
200
100
0
35
100
150
200
300
400
500
600
1000
2000
10380
[bp]
10,000 cells
5,000 cells
2,500 cells
1,250 cells
625 cells
NTC and cell supernatant

Figure 14B


Amplification
10,000 cells
2,000 cells
5,000 cells
1,250 cells
625 cells
NTC and cell supernatant
RFU (10^3)
15
10
5
0
0
10
20
30
40
Cycles

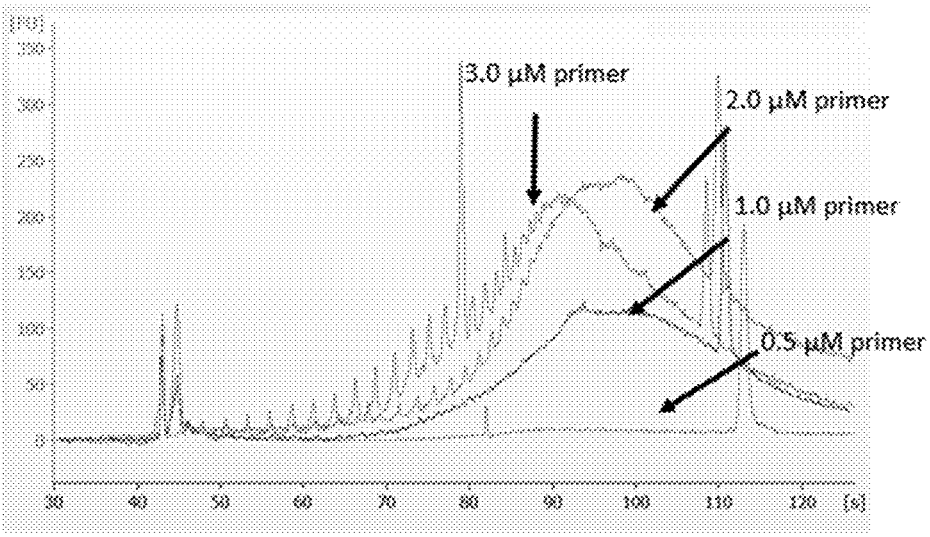
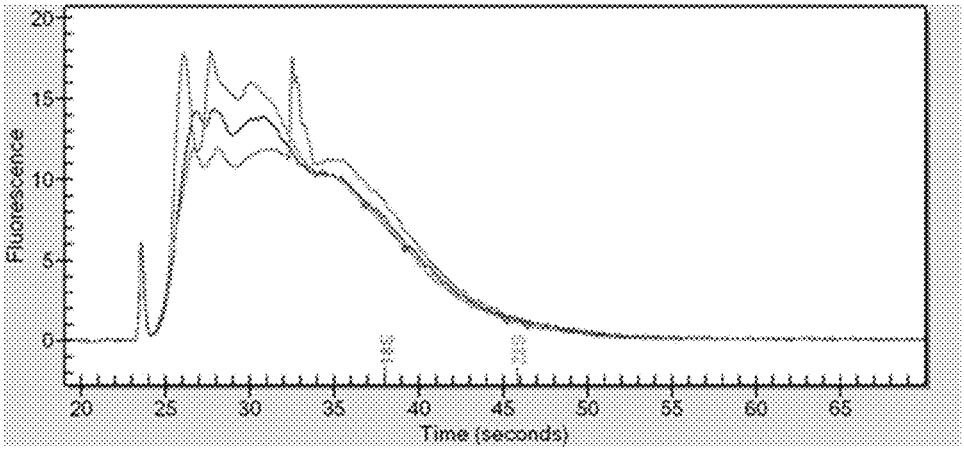


Figure 15


20
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Fluorescence
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Time (seconds)

Figure 16

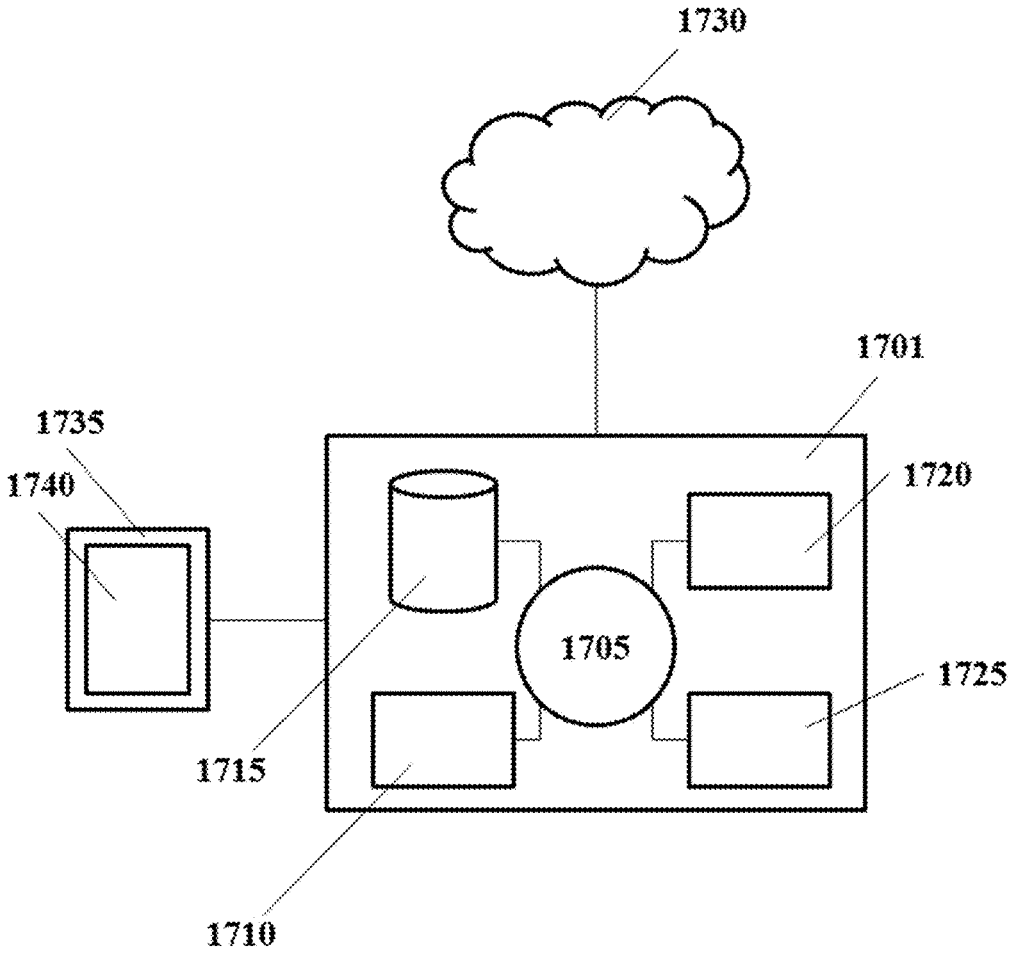

Figure 17

METHODS AND SYSTEMS FOR PROCESSING POLYNUCLEOTIDES

CROSS-REFERENCE TO RELATED APPLICATIONS

This application is a continuation-in-part of U.S. application Ser. No. 16/000,803, filed Jun. 5, 2018, which is a continuation of U.S. application Ser. No. 15/850,241, filed Dec 21, 2017, which is a continuation of U.S. patent application Ser. No. 15/588,519, filed May 5, 2017, now U.S. Pat No. 9,856,530, which is a continuation of U.S. patent application Ser. No. 15/376,582, filed Dec. 12, 2016, now U.S. Pat. No. 9,701,998, which is a continuation-in-part of U.S. patent application Ser. No. 14/104,650, filed on Dec. 12, 2013, now U.S. Pat. No. 9,567,631, which claims priority to U.S. Provisional Application No. 61/737,374, filed on Dec. 14, 2012; U.S. patent application Ser. No. 15/376,582 is also a continuation-in-part of U.S. patent application Ser. No. 14/250,701, filed on Apr. 11, 2014, which is a continuation of U.S. patent application Ser. No. 14/175,973, filed on Feb 7, 2014, now U.S. Pat. No. 9,388,465, which claims priority to U.S. Provisional Application No. 61/844,804, filed on Jul. 10, 2013, U.S. Provisional Application No. 61/840,403, filed on Jun. 27, 2013, U.S. Provisional Application No. 61/800,223, filed on Mar. 15, 2013, and U.S. Provisional Application No. 61/762,435, filed on Feb. 8, 2013, each of which is entirely incorporated herein by reference for all purposes. This application is also a continuation-in-part of U.S. application Ser. No. 15/598,898, filed May 18, 2017, which is a continuation of U.S. application Ser. No. 14/624,468, filed Feb 17, 2015, now U.S. Pat. No. 9,689,024, which is a divisional of U.S. patent application Ser. No. 13/966,150, filed Aug. 13, 2013, which claims priority to U.S. Provisional Patent Application No. 61/844,804, filed Jul. 10, 2013, U.S. Provisional Patent Application No. 61/840,403, filed Jun. 27, 2013, U.S. Provisional Patent Application No. 61/800,223, filed Mar. 15, 2013, U.S. Provisional Patent Application No. 61/762,435, filed Feb. 8, 2013, U.S. Provisional Patent Application No. 61/737,374, filed Dec. 14, 2012, and U.S. Provisional Patent Application No. 61/683,192, filed Aug. 14, 2012, each of which is entirely incorporated herein by reference for all purposes.

BACKGROUND

Significant advances in analyzing and characterizing biological and biochemical materials and systems have led to unprecedented advances in understanding the mechanisms of life, health, disease and treatment. Among these advances, technologies that target and characterize the genomic make up of biological systems have yielded some of the most groundbreaking results, including advances in the use and exploitation of genetic amplification technologies, and nucleic acid sequencing technologies.

Nucleic acid sequencing can be used to obtain information in a wide variety of biomedical contexts, including diagnostics, prognostics, biotechnology, and forensic biology. Sequencing may involve basic methods including Maxam-Gilbert sequencing and chain-termination methods, or de novo sequencing methods including shotgun sequencing and bridge PCR, or next-generation methods including polony sequencing, 454 pyrosequencing, Illumina sequencing, SOLiD sequencing, Ion Torrent semiconductor sequencing, HeliScope single molecule sequencing, SMRT® sequencing, and others.

Despite these advances in biological characterization, many challenges still remain unaddressed, or relatively poorly addressed by the solutions currently being offered. The present disclosure provides novel solutions and approaches to addressing many of the shortcomings of existing technologies.

BRIEF SUMMARY

Provided herein are methods, compositions and systems for analyzing individual cells or small populations of cells, including the analysis and attribution of nucleic acids from and to these individual cells or cell populations.

An aspect of the disclosure provides a method of analyzing nucleic acids from cells that includes providing nucleic acids derived from an individual cell into a discrete partition; generating one or more first nucleic acid sequences derived from the nucleic acids within the discrete partition, which one or more first nucleic acid sequences have attached thereto oligonucleotides that comprise a common nucleic acid barcode sequence; generating a characterization of the one or more first nucleic acid sequences or one or more second nucleic acid sequences derived from the one or more first nucleic acid sequences, which one or more second nucleic acid sequences comprise the common barcode sequence; and identifying the one or more first nucleic acid sequences or one or more second nucleic acid sequences as being derived from the individual cell based, at least in part, upon a presence of the common nucleic acid barcode sequence in the generated characterization.

In some embodiments, the discrete partition is a discrete droplet. In some embodiments, the oligonucleotides are co-partitioned with the nucleic acids derived from the individual cell into the discrete partition. In some embodiments, at least 10,000, at least 100,000 or at least 500,000 of the oligonucleotides are co-partitioned with the nucleic acids derived from the individual cell into the discrete partition.

In some embodiments, the oligonucleotides are provided attached to a bead, where each oligonucleotide on a bead comprises the same barcode sequence, and the bead is co-partitioned with the individual cell into the discrete partition. In some embodiments, the oligonucleotides are releasably attached to the bead. In some embodiments, the bead comprises a degradable bead. In some embodiments, prior to or during generating the one or more first nucleic acid sequences the method includes releasing the oligonucleotides from the bead via degradation of the bead. In some embodiments, prior to generating the characterization, the method includes releasing the one or more first nucleic acid sequences from the discrete partition.

In some embodiments, generating the characterization comprises sequencing the one or more first nucleic acid sequences or the one or more second nucleic acid sequences. The method may also include assembling a contiguous nucleic acid sequence for at least a portion of a genome of the individual cell from sequences of the one or more first nucleic acid sequences or the one or more second nucleic acid sequences. Moreover, the method may also include characterizing the individual cell based upon the nucleic acid sequence for at least a portion of the genome of the individual cell.

In some embodiments, the nucleic acids are released from the individual cell in the discrete partition. In some embodiments, the nucleic acids comprise ribonucleic acid (RNA), such as, for example, messenger RNA (mRNA). In some embodiments, generating one or more first nucleic acid sequences includes subjecting the nucleic acids to reverse

transcription under conditions that yield the one or more first nucleic acid sequences. In some embodiments, the reverse transcription occurs in the discrete partition. In some embodiments, the oligonucleotides are provided in the discrete partition and include a poly-T sequence. In some embodiments, the reverse transcription comprises hybridizing the poly-T sequence to at least a portion of each of the nucleic acids and extending the poly-T sequence in template directed fashion. In some embodiments, the oligonucleotides include an anchoring sequence that facilitates hybridization of the poly-T sequence. In some embodiments, the oligonucleotides include a random priming sequence that can be, for example, a random hexamer. In some embodiments, the reverse transcription comprises hybridizing the random priming sequence to at least a portion of each of the nucleic acids and extending the random priming sequence in template directed fashion.

In some embodiments, a given one of the one or more first nucleic acid sequences has sequence complementarity to at least a portion of a given one of the nucleic acids. In some embodiments, the discrete partition at most includes the individual cell among a plurality of cells. In some embodiments, the oligonucleotides include a unique molecular sequence segment. In some embodiments, the method can include identifying an individual nucleic acid sequence of the one or more first nucleic acid sequences or of the one or more second nucleic acid sequences as derived from a given nucleic acid of the nucleic acids based, at least in part, upon a presence of the unique molecular sequence segment. In some embodiments, the method includes determining an amount of the given nucleic acid based upon a presence of the unique molecular sequence segment.

In some embodiments, the method includes, prior to generating the characterization, adding one or more additional sequences to the one or more first nucleic acid sequences to generate the one or more second nucleic acid sequences. In some embodiments, the method includes adding a first additional nucleic acid sequence to the one or more first nucleic acid sequences with the aid of a switch oligonucleotide. In some embodiments, the switch oligonucleotide hybridizes to at least a portion of the one or more first nucleic acid sequences and is extended in a template directed fashion to couple the first additional nucleic acid sequence to the one or more first nucleic acid sequences. In some embodiments, the method includes amplifying the one of more first nucleic acid sequences coupled to the first additional nucleic acid sequence. In some embodiments, the amplifying occurs in the discrete partition. In some embodiments, the amplifying occurs after releasing the one or more first nucleic acid sequences coupled to the first additional nucleic acid sequence from the discrete partition.

In some embodiments, after the amplifying, the method includes adding one or more second additional nucleic acid sequences to the one or more first nucleic acid sequences coupled to the first additional sequence to generate the one or more second nucleic acid sequences. In some embodiments, the adding the one or more second additional sequences includes removing a portion of each of the one or more first nucleic acid sequences coupled to the first additional nucleic acid sequence and coupling thereto the one or more second additional nucleic acid sequences. In some embodiments, the removing is completed via shearing of the one or more first nucleic acid sequences coupled (e.g., ligated) to the first additional nucleic acid sequence.

In some embodiments, prior to generating the characterization, the method includes subjecting the one or more first nucleic acid sequences to transcription to generate one or more RNA fragments. In some embodiments, the transcription occurs after releasing the one or more first nucleic acid sequences from the discrete partition. In some embodiments, the oligonucleotides include a T7 promoter sequence. In some embodiments, prior to generating the characterization, the method includes removing a portion of each of the one or more RNA sequences and coupling an additional sequence to the one or more RNA sequences. In some embodiments, prior to generating the characterization, the method includes subjecting the one or more RNA sequences coupled to the additional sequence to reverse transcription to generate the one or more second nucleic acid sequences. In some embodiments, prior to generating the characterization, the method includes amplifying the one or more second nucleic acid sequences. In some embodiments, prior to generating the characterization, the method includes subjecting the one or more RNA sequences to reverse transcription to generate one or more DNA sequences. In some embodiments, prior to generating the characterization, the method includes removing a portion of each of the one or more DNA sequences and coupling one or more additional sequences to the one or more DNA sequences to generate the one or more second nucleic acid sequences. In some embodiments, prior to generating the characterization, the method includes amplifying the one or more second nucleic acid sequences.

In some embodiments, the nucleic acids include complementary (cDNA) generated from reverse transcription of RNA from the individual cell. In some embodiments, the oligonucleotides include a priming sequence and are provided in the discrete partition. In some embodiments, the priming sequence includes a random N-mer. In some embodiments, generating the one or more first nucleic acid sequences includes hybridizing the priming sequence to the cDNA and extending the priming sequence in template directed fashion.

In some embodiments, the discrete partition includes switch oligonucleotides comprising a complement sequence of the oligonucleotides. In some embodiments, generating the one or more first nucleic acid sequences includes hybridizing the switch oligonucleotides to at least a portion of nucleic acid fragments derived from the nucleic acids and extending the switch oligonucleotides in template directed fashion. In some embodiments, generating the one or more first nucleic acid sequences includes attaching the oligonucleotides to the one or more first nucleic acid sequences. In some embodiments, the one or more first nucleic acid sequences are nucleic acid fragments derived from the nucleic acids. In some embodiments, generating the one or more first nucleic acid sequences includes coupling (e.g., ligating) the oligonucleotides to the nucleic acids.

In some embodiments, a plurality of partitions comprises the discrete partition. In some embodiments, the plurality of partitions, on average, comprises less than one cell per partition. In some embodiments, less than 25% of partitions of the plurality of partitions do not comprise a cell. In some embodiments, the plurality of partitions comprises discrete partitions each having at least one partitioned cell. In some embodiments, fewer than 25%, fewer than 20%, fewer than 15%, fewer than 10%, fewer than 5% or fewer than 1% of the discrete partitions comprise more than one cell. In some embodiments, at least a subset of the discrete partitions comprises a bead. In some embodiments, at least 75%, at least 80%, at least 85%, at least 90%, at least 95% or at least 99% of the discrete partitions comprise at least one cell and at least one bead. In some embodiments, the discrete partitions include partitioned nucleic acid barcode sequences. In

some embodiments, the discrete partitions include at least 1,000, at least 10,000, or at least 100,000 different partitioned nucleic acid barcode sequences. In some embodiments, the plurality of partitions comprises at least 1,000, at least 10,000 or at least 100,000 partitions.

In another aspect, the disclosure provides a method of characterizing cells in a population of a plurality of different cell types that includes providing nucleic acids from individual cells in the population into discrete partitions; attaching oligonucleotides that comprise a common nucleic acid barcode sequence to one or more fragments of the nucleic acids from the individual cells within the discrete partitions, where a plurality of different partitions comprise different common nucleic acid barcode sequences; and characterizing the one or more fragments of the nucleic acids from the plurality of discrete partitions, and attributing the one or more fragments to individual cells based, at least in part, upon the presence of a common barcode sequence; and characterizing a plurality of individual cells in the population based upon the characterization of the one or more fragments in the plurality of discrete partitions.

In some embodiments, the method includes fragmenting the nucleic acids. In some embodiments, the discrete partitions are droplets. In some embodiments, the characterizing the one or more fragments of the nucleic acids includes sequencing ribosomal deoxyribonucleic acid from the individual cells, and the characterizing the cells comprises identifying a cell genus, species, strain or variant. In some embodiments, the individual cells are derived from a microbiome sample. In some embodiments, the individual cells are derived from a human tissue sample. In some embodiments, the individual cells are derived from circulating cells in a mammal. In some embodiments, the individual cells are derived from a forensic sample. In some embodiments, the nucleic acids are released from the individual cells in the discrete partitions.

An additional aspect of the disclosure provides a method of characterizing an individual cell or population of cells that includes incubating a cell with a plurality of different cell surface feature binding group types, where each different cell surface binding group type is capable of binding to a different cell surface feature, and where each different cell surface binding group type comprises a reporter oligonucleotide associated therewith, under conditions that allow binding between one or more cell surface feature binding groups and its respective cell surface feature, if present; partitioning the cell into a partition that comprises a plurality of oligonucleotides comprising a barcode sequence; attaching the barcode sequence to oligonucleotide reporter groups present in the partition; sequencing the oligonucleotide reporter groups and attached barcodes; and characterizing cell surface features present on the cell based upon reporter oligonucleotides that are sequenced.

An additional aspect of the disclosure provides a composition comprising a plurality of partitions, each of the plurality of partitions comprising an individual cell and a population of oligonucleotides that comprise a common nucleic acid barcode sequence. In some embodiments, the plurality of partitions comprises droplets in an emulsion. In some embodiments, the population of oligonucleotides within each of the plurality of partitions is coupled to a bead disposed within each of the plurality of partitions. In some embodiments, the individual cell has associated therewith a plurality of different cell surface feature binding groups associated with their respective cell surface features and each different type of cell surface feature binding group includes an oligonucleotide reporter group comprising a different nucleotide sequence. In some embodiments, the plurality of different cell surface feature binding groups includes a plurality of different antibodies or antibody fragments having a binding affinity for a plurality of different cell surface features.

Additional aspects and advantages of the present disclosure will become readily apparent to those skilled in the art from the following detailed description, wherein only illustrative embodiments of the present disclosure are shown and described. As will be realized, the present disclosure is capable of other and different embodiments, and its several details are capable of modifications in various obvious respects, all without departing from the disclosure. Accordingly, the drawings and description are to be regarded as illustrative in nature, and not as restrictive.

INCORPORATION BY REFERENCE

All publications, patents, and patent applications mentioned in this specification are herein incorporated by reference to the same extent as if each individual publication, patent, or patent application was specifically and individually indicated to be incorporated by reference. To the extent publications and patents or patent applications incorporated by reference contradict the disclosure contained in the specification, the specification is intended to supersede and/or take precedence over any such contradictory material.

BRIEF DESCRIPTION OF THE DRAWINGS

The novel features of the invention are set forth with particularity in the appended claims. A better understanding of the features and advantages of the present invention will be obtained by reference to the following detailed description that sets forth illustrative embodiments, in which the principles of the invention are utilized, and the accompanying drawings (also "Figure" and "FIG." herein), of which:

FIG. **1** schematically illustrates a microfluidic channel structure for partitioning individual or small groups of cells.

FIG. **2** schematically illustrates a microfluidic channel structure for co-partitioning cells and beads or microcapsules comprising additional reagents.

FIG. **3** schematically illustrates an example process for amplification and barcoding of cell's nucleic acids.

FIG. **4** provides a schematic illustration of use of barcoding of cell's nucleic acids in attributing sequence data to individual cells or groups of cells for use in their characterization.

FIG. **5** provides a schematic illustrating cells associated with labeled cell-binding ligands.

FIG. **6** provides a schematic illustration of an example workflow for performing RNA analysis using the methods described herein.

FIG. **7** provides a schematic illustration of an example barcoded oligonucleotide structure for use in analysis of ribonucleic (RNA) using the methods described herein.

FIG. **8** provides an image of individual cells co-partitioned along with individual barcode bearing beads

FIG. **9**A-E provides schematic illustration of example barcoded oligonucleotide structures for use in analysis of RNA and example operations for performing RNA analysis.

FIG. **10** provides schematic illustration of example barcoded oligonucleotide structure for use in example analysis of RNA and use of a sequence for in vitro transcription.

FIG. **11** provides schematic illustration of an example barcoded oligonucleotide structure for use in analysis of RNA and example operations for performing RNA analysis.

FIG. **12**A-B provides schematic illustration of example barcoded oligonucleotide structure for use in analysis of RNA.

FIG. **13**A-C provides illustrations of example yields from template switch reverse transcription and PCR in partitions.

FIG. **14**A-B provides illustrations of example yields from reverse transcription and cDNA amplification in partitions with various cell numbers.

FIG. **15** provides an illustration of example yields from cDNA synthesis and real-time quantitative PCR at various input cell concentrations and also the effect of varying primer concentration on yield at a fixed cell input concentration.

FIG. **16** provides an illustration of example yields from in vitro transcription.

FIG. **17** shows an example computer control system that is programmed or otherwise configured to implement methods provided herein.

DETAILED DESCRIPTION

While various embodiments of the invention have been shown and described herein, it will be obvious to those skilled in the art that such embodiments are provided by way of example only. Numerous variations, changes, and substitutions may occur to those skilled in the art without departing from the invention. It should be understood that various alternatives to the embodiments of the invention described herein may be employed.

Where values are described as ranges, it will be understood that such disclosure includes the disclosure of all possible sub-ranges within such ranges, as well as specific numerical values that fall within such ranges irrespective of whether a specific numerical value or specific sub-range is expressly stated.

I. Single Cell Analysis

Advanced nucleic acid sequencing technologies have yielded monumental results in sequencing biological materials, including providing substantial sequence information on individual organisms, and relatively pure biological samples. However, these systems have not proven effective at being able to identify and characterize sub-populations of cells in biological samples that may represent a smaller minority of the overall make up of the sample, but for which individualized sequence information could prove even more valuable.

Most nucleic acid sequencing technologies derive the nucleic acids that they sequence from collections of cells derived from tissue or other samples. The cells can be processed, en masse, to extract the genetic material that represents an average of the population of cells, which can then be processed into sequencing ready DNA libraries that are configured for a given sequencing technology. As will be appreciated, although often discussed in terms of DNA or nucleic acids, the nucleic acids derived from the cells may include DNA, or RNA, including, e.g., mRNA, total RNA, or the like, that may be processed to produce cDNA for sequencing, e.g., using any of a variety of RNA-seq methods. Following from this processing, absent a cell specific marker, attribution of genetic material as being contributed by a subset of cells or all cells in a sample is virtually impossible in such an ensemble approach.

In addition to the inability to attribute characteristics to particular subsets of populations of cells, such ensemble sample preparation methods also are, from the outset, predisposed to primarily identifying and characterizing the majority constituents in the sample of cells, and are not designed to be able to pick out the minority constituents, e.g., genetic material contributed by one cell, a few cells, or a small percentage of total cells in the sample. Likewise, where analyzing expression levels, e.g., of mRNA, an ensemble approach would be predisposed to presenting potentially grossly inaccurate data from cell populations that are non-homogeneous in terms of expression levels. In some cases, where expression is high in a small minority of the cells in an analyzed population, and absent in the majority of the cells of the population, an ensemble method would indicate low level expression for the entire population.

This original majority bias is further magnified, and even overwhelming, through processing operations used in building up the sequencing libraries from these samples. In particular, most next generation sequencing technologies rely upon the geometric amplification of nucleic acid fragments, such as the polymerase chain reaction, in order to produce sufficient DNA for the sequencing library. However, such geometric amplification is biased toward amplification of majority constituents in a sample, and may not preserve the starting ratios of such minority and majority components. By way of example, if a sample includes 95% DNA from a particular cell type in a sample, e.g., host tissue cells, and 5% DNA from another cell type, e.g., cancer cells, PCR based amplification can preferentially amplify the majority DNA in place of the minority DNA, both as a function of comparative exponential amplification (the repeated doubling of the higher concentration quickly outpaces that of the smaller fraction) and as a function of sequestration of amplification reagents and resources (as the larger fraction is amplified, it preferentially utilizes primers and other amplification reagents).

While some of these difficulties may be addressed by utilizing different sequencing systems, such as single molecule systems that don't require amplification, the single molecule systems, as well as the ensemble sequencing methods of other next generation sequencing systems, can also have requirements for sufficiently large input DNA requirements. In particular, single molecule sequencing systems like the Pacific Biosciences SMRT Sequencing system can have sample input DNA requirements of from 500 nanograms (ng) to upwards of 10 micrograms (μg), which is far larger than what can be derived from individual cells or even small subpopulations of cells. Likewise, other NGS systems can be optimized for starting amounts of sample DNA in the sample of from approximately 50 ng to about 1 μg.

II. Compartmentalization and Characterization of Cells

Disclosed herein, however, are methods and systems for characterizing nucleic acids from small populations of cells, and in some cases, for characterizing nucleic acids from individual cells, especially in the context of larger populations of cells. The methods and systems provide advantages of being able to provide the attribution advantages of the non-amplified single molecule methods with the high throughput of the other next generation systems, with the additional advantages of being able to process and sequence extremely low amounts of input nucleic acids derivable from individual cells or small collections of cells.

In particular, the methods described herein compartmentalize the analysis of individual cells or small populations of cells, including e.g., nucleic acids from individual cells or small groups of cells, and then allow that analysis to be attributed back to the individual cell or small group of cells

from which the nucleic acids were derived. This can be accomplished regardless of whether the cell population represents a 50/50 mix of cell types, a 90/10 mix of cell types, or virtually any ratio of cell types, as well as a complete heterogeneous mix of different cell types, or any mixture between these. Differing cell types may include cells or biologic organisms from different tissue types of an individual, from different individuals, from differing genera, species, strains, variants, or any combination of any or all of the foregoing. For example, differing cell types may include normal and tumor tissue from an individual, multiple different bacterial species, strains and/or variants from environmental, forensic, microbiome or other samples, or any of a variety of other mixtures of cell types.

In one aspect, the methods and systems described herein, provide for the compartmentalization, depositing or partitioning of the nucleic acid contents of individual cells from a sample material containing cells, into discrete compartments or partitions (referred to interchangeably herein as partitions), where each partition maintains separation of its own contents from the contents of other partitions. Unique identifiers, e.g., barcodes, may be previously, subsequently or concurrently delivered to the partitions that hold the compartmentalized or partitioned cells, in order to allow for the later attribution of the characteristics of the individual cells to the particular compartment.

As used herein, in some aspects, the partitions refer to containers or vessels (such as wells, microwells, tubes, through ports in nanoarray substrates, e.g., BioTrove nanoarrays, or other containers). In many some aspects, however, the compartments or partitions comprise partitions that are flowable within fluid streams. These partitions may be comprised of, e.g., microcapsules or micro-vesicles that have an outer barrier surrounding an inner fluid center or core, or they may be a porous matrix that is capable of entraining and/or retaining materials within its matrix. In some aspects, however, these partitions comprise droplets of aqueous fluid within a non-aqueous continuous phase, e.g., an oil phase. A variety of different vessels are described in, for example, U.S. patent application Ser. No. 13/966,150, filed Aug. 13, 2013, published as U.S. Patent Publication No. 2014/0155295, the full disclosure of which is incorporated herein by reference in its entirety for all purposes. Likewise, emulsion systems for creating stable droplets in non-aqueous or oil continuous phases are described in detail in, e.g., U.S. Patent Publication No. 2010/0105112, the full disclosure of which is incorporated herein by reference in its entirety for all purposes.

In the case of droplets in an emulsion, allocating individual cells to discrete partitions may generally be accomplished by introducing a flowing stream of cells in an aqueous fluid into a flowing stream of a non-aqueous fluid, such that droplets are generated at the junction of the two streams. By providing the aqueous cell-containing stream at a certain concentration level of cells, one can control the level of occupancy of the resulting partitions in terms of numbers of cells. In some cases, where single cell partitions are desired, it may be desirable to control the relative flow rates of the fluids such that, on average, the partitions contain less than one cell per partition, in order to ensure that those partitions that are occupied, are primarily singly occupied. Likewise, one may wish to control the flow rate to provide that a higher percentage of partitions are occupied, e.g., allowing for only a small percentage of unoccupied partitions. In some aspects, the flows and channel architectures are controlled as to ensure a desired number of singly occupied partitions, less than a certain level of unoccupied partitions and less than a certain level of multiply occupied partitions.

In many cases, the systems and methods are used to ensure that the substantial majority of occupied partitions (partitions containing one or more microcapsules) include no more than 1 cell per occupied partition. In some cases, the partitioning process is controlled such that fewer than 25% of the occupied partitions contain more than one cell, and in many cases, fewer than 20% of the occupied partitions have more than one cell, while in some cases, fewer than 10% or even fewer than 5% of the occupied partitions include more than one cell per partition.

Additionally or alternatively, in many cases, it is desirable to avoid the creation of excessive numbers of empty partitions. While this may be accomplished by providing sufficient numbers of cells into the partitioning zone, the poissonian distribution would expectedly increase the number of partitions that would include multiple cells. As such, in accordance with aspects described herein, the flow of one or more of the cells, or other fluids directed into the partitioning zone are controlled such that, in many cases, no more than 50% of the generated partitions are unoccupied, i.e., including less than 1 cell, no more than 25% of the generated partitions, no more than 10% of the generated partitions, may be unoccupied. Further, in some aspects, these flows are controlled so as to present non-poissonian distribution of single occupied partitions while providing lower levels of unoccupied partitions. Restated, in some aspects, the above noted ranges of unoccupied partitions can be achieved while still providing any of the single occupancy rates described above. For example, in many cases, the use of the systems and methods described herein creates resulting partitions that have multiple occupancy rates of from less than 25%, less than 20%, less than 15%, less than 10%, and in many cases, less than 5%, while having unoccupied partitions of from less than 50%, less than 40%, less than 30%, less than 20%, less than 10%, and in some cases, less than 5%.

As will be appreciated, the above-described occupancy rates are also applicable to partitions that include both cells and beads carrying the barcode oligonucleotides. In particular, in some aspects, a substantial percentage of the overall occupied partitions will include both a bead and a cell. In particular, it may be desirable to provide that at least 50% of the partitions are occupied by at least one cell and at least one bead, or at least 75% of the partitions may be so occupied, or even at least 80% or at least 90% of the partitions may be so occupied. Further, in those cases where it is desired to provide a single cell and a single bead within a partition, at least 50% of the partitions can be so occupied, at least 60%, at least 70%, at least 80% or even at least 90% of the partitions can be so occupied.

Although described in terms of providing substantially singly occupied partitions, above, in certain cases, it is desirable to provide multiply occupied partitions, e.g., containing two, three, four or more cells and/or beads within a single partition. Accordingly, as noted above, the flow characteristics of the cell and/or bead containing fluids and partitioning fluids may be controlled to provide for such multiply occupied partitions. In particular, the flow parameters may be controlled to provide a desired occupancy rate at greater than 50% of the partitions, greater than 75%, and in some cases greater than 80%, 90%, 95%, or higher.

Additionally, in many cases, the multiple beads within a single partition may comprise different reagents associated therewith. In such cases, it may be advantageous to introduce different beads into a common channel or droplet

generation junction, from different bead sources, i.e., containing different associated reagents, through different channel inlets into such common channel or droplet generation junction. In such cases, the flow and frequency of the different beads into the channel or junction may be controlled to provide for the desired ratio of microcapsules from each source, while ensuring the desired pairing or combination of such beads into a partition with the desired number of cells.

The partitions described herein are often characterized by having extremely small volumes, e.g., less than 10 μL, less than 5 μL, less than 1 μL, less than 900 picoliters (pL), less than 800 pL, less than 700 pL, less than 600 pL, less than 500 pL, less than 400 pL, less than 300 pL, less than 200 pL, less than 100 pL, less than 50 pL, less than 20 pL, less than 10 pL, less than 1 pL, less than 500 nanoliters (nL), or even less than 100 nL, 50 nL, or even less.

For example, in the case of droplet based partitions, the droplets may have overall volumes that are less than 1000 pL, less than 900 pL, less than 800 pL, less than 700 pL, less than 600 pL, less than 500 pL, less than 400 pL, less than 300 pL, less than 200 pL, less than 100 pL, less than 50 pL, less than 20 pL, less than 10 pL, or even less than 1 pL. Where co-partitioned with beads, it will be appreciated that the sample fluid volume, e.g., including co-partitioned cells, within the partitions may be less than 90% of the above described volumes, less than 80%, less than 70%, less than 60%, less than 50%, less than 40%, less than 30%, less than 20%, or even less than 10% the above described volumes.

As is described elsewhere herein, partitioning species may generate a population of partitions. In such cases, any suitable number of partitions can be generated to generate the population of partitions. For example, in a method described herein, a population of partitions may be generated that comprises at least about 1,000 partitions, at least about 5,000 partitions, at least about 10,000 partitions, at least about 50,000 partitions, at least about 100,000 partitions, at least about 500,000 partitions, at least about 1,000,000 partitions, at least about 5,000,000 partitions at least about 10,000,000 partitions, at least about 50,000,000 partitions, at least about 100,000,000 partitions, at least about 500,000,000 partitions or at least about 1,000,000,000 partitions. Moreover, the population of partitions may comprise both unoccupied partitions (e.g., empty partitions) and occupied partitions

In certain cases, microfluidic channel networks are particularly suited for generating partitions as described herein. Examples of such microfluidic devices include those described in detail in Provisional U.S. Patent Application No. 61/977,804, filed Apr. 4, 2014, the full disclosure of which is incorporated herein by reference in its entirety for all purposes. Alternative mechanisms may also be employed in the partitioning of individual cells, including porous membranes through which aqueous mixtures of cells are extruded into non-aqueous fluids. Such systems are generally available from, e.g., Nanomi, Inc.

An example of a simplified microfluidic channel structure for partitioning individual cells is illustrated in FIG. **1**. As described elsewhere herein, in some cases, the majority of occupied partitions include no more than one cell per occupied partition and, in some cases, some of the generated partitions are unoccupied. In some cases, though, some of the occupied partitions may include more than one cell. In some cases, the partitioning process may be controlled such that fewer than 25% of the occupied partitions contain more than one cell, and in many cases, fewer than 20% of the occupied partitions have more than one cell, while in some cases, fewer than 10% or even fewer than 5% of the occupied partitions include more than one cell per partition. As shown, the channel structure can include channel segments **102**, **104**, **106** and **108** communicating at a channel junction **110**. In operation, a first aqueous fluid **112** that includes suspended cells **114**, may be transported along channel segment **102** into junction **110**, while a second fluid **116** that is immiscible with the aqueous fluid **112** is delivered to the junction **110** from channel segments **104** and **106** to create discrete droplets **118** of the aqueous fluid including individual cells **114**, flowing into channel segment **108**.

In some aspects, this second fluid **116** comprises an oil, such as a fluorinated oil, that includes a fluorosurfactant for stabilizing the resulting droplets, e.g., inhibiting subsequent coalescence of the resulting droplets. Examples of particularly useful partitioning fluids and fluorosurfactants are described for example, in U.S. Patent Publication No. 2010/0105112, the full disclosure of which is hereby incorporated herein by reference in its entirety for all purposes.

In other aspects, in addition to or as an alternative to droplet based partitioning, cells may be encapsulated within a microcapsule that comprises an outer shell or layer or porous matrix in which is entrained one or more individual cells or small groups of cells, and may include other reagents. Encapsulation of cells may be carried out by a variety of processes. In general, such processes combine an aqueous fluid containing the cells to be analyzed with a polymeric precursor material that may be capable of being formed into a gel or other solid or semi-solid matrix upon application of a particular stimulus to the polymer precursor. Such stimuli include, e.g., thermal stimuli (either heating or cooling), photo-stimuli (e.g., through photo-curing), chemical stimuli (e.g., through crosslinking, polymerization initiation of the precursor (e.g., through added initiators), or the like.

Preparation of microcapsules comprising cells may be carried out by a variety of methods. For example, air knife droplet or aerosol generators may be used to dispense droplets of precursor fluids into gelling solutions in order to form microcapsules that include individual cells or small groups of cells. Likewise, membrane based encapsulation systems, such as those available from, e.g., Nanomi, Inc., may be used to generate microcapsules as described herein. In some aspects, microfluidic systems like that shown in FIG. **1** may be readily used in encapsulating cells as described herein. In particular, and with reference to FIG. **1**, the aqueous fluid comprising the cells and the polymer precursor material is flowed into channel junction **110**, where it is partitioned into droplets **118** comprising the individual cells **114**, through the flow of non-aqueous fluid **116**. In the case of encapsulation methods, non-aqueous fluid **116** may also include an initiator to cause polymerization and/or crosslinking of the polymer precursor to form the microcapsule that includes the entrained cells. Examples of particularly useful polymer precursor/initiator pairs include those described in, e.g., U.S. Patent Application Nos. 61/940,318, filed Feb. 7, 2014, 61/991,018, Filed May 9, 2014, and U.S. patent application Ser. No. 14/316,383, filed Jun. 26, 2014, published as U.S. Patent Publication No. 2014/0378345, the full disclosures of which are hereby incorporated herein by reference in their entireties for all purposes.

For example, in the case where the polymer precursor material comprises a linear polymer material, e.g., a linear polyacrylamide, PEG, or other linear polymeric material, the activation agent may comprise a cross-linking agent, or a chemical that activates a cross-linking agent within the

formed droplets. Likewise, for polymer precursors that comprise polymerizable monomers, the activation agent may comprise a polymerization initiator. For example, in certain cases, where the polymer precursor comprises a mixture of acrylamide monomer with a N,N'-bis-(acryloyl) cystamine (BAC) comonomer, an agent such as tetraethylmethylenediamine (TEMED) may be provided within the second fluid streams in channel segments **104** and **106**, which initiates the copolymerization of the acrylamide and BAC into a cross-linked polymer network or, hydrogel.

Upon contact of the second fluid stream **116** with the first fluid stream **112** at junction **110** in the formation of droplets, the TEMED may diffuse from the second fluid **116** into the aqueous first fluid **112** comprising the linear polyacrylamide, which will activate the crosslinking of the polyacrylamide within the droplets, resulting in the formation of the gel, e.g., hydrogel, microcapsules **118**, as solid or semi-solid beads or particles entraining the cells **114**. Although described in terms of polyacrylamide encapsulation, other 'activatable' encapsulation compositions may also be employed in the context of the methods and compositions described herein. For example, formation of alginate droplets followed by exposure to divalent metal ions, e.g., Ca2+, can be used as an encapsulation process using the described processes. Likewise, agarose droplets may also be transformed into capsules through temperature based gelling, e.g., upon cooling, or the like. As will be appreciated, in some cases, encapsulated cells can be selectively releasable from the microcapsule, e.g., through passage of time, or upon application of a particular stimulus, that degrades the microcapsule sufficiently to allow the cell, or its contents to be released from the microcapsule, e.g., into an additional partition, such as a droplet. For example, in the case of the polyacrylamide polymer described above, degradation of the microcapsule may be accomplished through the introduction of an appropriate reducing agent, such as DTT or the like, to cleave disulfide bonds that cross link the polymer matrix (See, e.g., U.S. Provisional Patent Application Nos. 61/940,318, filed Feb. 7, 2014, 61/991,018, Filed May 9, 2014, and U.S. patent application Ser. No. 14/316,383, filed Jun. 26, 2014, published as U.S. Patent Publication No. 2014/0378345, the full disclosures of which are hereby incorporated herein by reference in their entirety for all purposes.

As will be appreciated, encapsulated cells or cell populations provide certain potential advantages of being storable, and more portable than droplet based partitioned cells. Furthermore, in some cases, it may be desirable to allow cells to be analyzed to incubate for a select period of time, in order to characterize changes in such cells over time, either in the presence or absence of different stimuli. In such cases, encapsulation of individual cells may allow for longer incubation than simple partitioning in emulsion droplets, although in some cases, droplet partitioned cells may also be incubated form different periods of time, e.g., at least 10 seconds, at least 30 seconds, at least 1 minute, at least 5 minutes, at least 10 minutes, at least 30 minutes, at least 1 hour, at least 2 hours, at least 5 hours, or at least 10 hours or more. As alluded to above, the encapsulation of cells may constitute the partitioning of the cells into which other reagents are co-partitioned. Alternatively, encapsulated cells may be readily deposited into other partitions, e.g., droplets, as described above.

In accordance with certain aspects, the cells may be partitioned along with lysis reagents in order to release the contents of the cells within the partition. In such cases, the lysis agents can be contacted with the cell suspension concurrently with, or immediately prior to the introduction of the cells into the partitioning junction/droplet generation zone, e.g., through an additional channel or channels upstream of channel junction **110**. Examples of lysis agents include bioactive reagents, such as lysis enzymes that are used for lysis of different cell types, e.g., gram positive or negative bacteria, plants, yeast, mammalian, etc., such as lysozymes, achromopeptidase, lysostaphin, labiase, kitalase, lyticase, and a variety of other lysis enzymes available from, e.g., Sigma-Aldrich, Inc. (St Louis, Mo.), as well as other commercially available lysis enzymes. Other lysis agents may additionally or alternatively be co-partitioned with the cells to cause the release of the cell's contents into the partitions. For example, in some cases, surfactant based lysis solutions may be used to lyse cells, although these may be less desirable for emulsion based systems where the surfactants can interfere with stable emulsions. In some cases, lysis solutions may include non-ionic surfactants such as, for example, TritonX-100 and Tween 20. In some cases, lysis solutions may include ionic surfactants such as, for example, sarcosyl and sodium dodecyl sulfate (SDS). Similarly, lysis methods that employ other methods may be used, such as electroporation, thermal, acoustic or mechanical cellular disruption may also be used in certain cases, e.g., non-emulsion based partitioning such as encapsulation of cells that may be in addition to or in place of droplet partitioning, where any pore size of the encapsulate is sufficiently small to retain nucleic acid fragments of a desired size, following cellular disruption.

In addition to the lysis agents co-partitioned with the cells described above, other reagents can also be co-partitioned with the cells, including, for example, DNase and RNase inactivating agents or inhibitors, such as proteinase K, chelating agents, such as EDTA, and other reagents employed in removing or otherwise reducing negative activity or impact of different cell lysate components on subsequent processing of nucleic acids. In addition, in the case of encapsulated cells, the cells may be exposed to an appropriate stimulus to release the cells or their contents from a co-partitioned microcapsule. For example, in some cases, a chemical stimulus may be co-partitioned along with an encapsulated cell to allow for the degradation of the microcapsule and release of the cell or its contents into the larger partition. In some cases, this stimulus may be the same as the stimulus described elsewhere herein for release of oligonucleotides from their respective bead or partition. In alternative aspects, this may be a different and non-overlapping stimulus, in order to allow an encapsulated cell to be released into a partition at a different time from the release of oligonucleotides into the same partition.

Additional reagents may also be co-partitioned with the cells, such as endonucleases to fragment the cell's DNA, DNA polymerase enzymes and dNTPs used to amplify the cell's nucleic acid fragments and to attach the barcode oligonucleotides to the amplified fragments. Additional reagents may also include reverse transcriptase enzymes, including enzymes with terminal transferase activity, primers and oligonucleotides, and switch oligonucleotides (also referred to herein as "switch oligos") which can be used for template switching. In some cases, template switching can be used to increase the length of a cDNA. In one example of template switching, cDNA can be generated from reverse transcription of a template, e.g., cellular mRNA, where a reverse transcriptase with terminal transferase activity can add additional nucleotides, e.g., polyC, to the cDNA that are not encoded by the template, such, as at an end of the cDNA. Switch oligos can include sequences complementary to the additional nucleotides, e.g. polyG. The additional nucleo-

tides (e.g., polyC) on the cDNA can hybridize to the sequences complementary to the additional nucleotides (e.g., polyG) on the switch oligo, whereby the switch oligo can be used by the reverse transcriptase as template to further extend the cDNA. Switch oligos may comprise deoxyribonucleic acids, ribonucleic acids, modified nucleic acids including locked nucleic acids (LNA), or any combination.

In some cases, the length of a switch oligo may be 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99, 100, 101, 102, 103, 104, 105, 106, 107, 108, 109, 110, 111, 112, 113, 114, 115, 116, 117, 118, 119, 120, 121, 122, 123, 124, 125, 126, 127, 128, 129, 130, 131, 132, 133, 134, 135, 136, 137, 138, 139, 140, 141, 142, 143, 144, 145, 146, 147, 148, 149, 150, 151, 152, 153, 154, 155, 156, 157, 158, 159, 160, 161, 162, 163, 164, 165, 166, 167, 168, 169, 170, 171, 172, 173, 174, 175, 176, 177, 178, 179, 180, 181, 182, 183, 184, 185, 186, 187, 188, 189, 190, 191, 192, 193, 194, 195, 196, 197, 198, 199, 200, 201, 202, 203, 204, 205, 206, 207, 208, 209, 210, 211, 212, 213, 214, 215, 216, 217, 218, 219, 220, 221, 222, 223, 224, 225, 226, 227, 228, 229, 230, 231, 232, 233, 234, 235, 236, 237, 238, 239, 240, 241, 242, 243, 244, 245, 246, 247, 248, 249, 250 nucleotides or longer.

In some cases, the length of a switch oligo may be at least 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99, 100, 101, 102, 103, 104, 105, 106, 107, 108, 109, 110, 111, 112, 113, 114, 115, 116, 117, 118, 119, 120, 121, 122, 123, 124, 125, 126, 127, 128, 129, 130, 131, 132, 133, 134, 135, 136, 137, 138, 139, 140, 141, 142, 143, 144, 145, 146, 147, 148, 149, 150, 151, 152, 153, 154, 155, 156, 157, 158, 159, 160, 161, 162, 163, 164, 165, 166, 167, 168, 169, 170, 171, 172, 173, 174, 175, 176, 177, 178, 179, 180, 181, 182, 183, 184, 185, 186, 187, 188, 189, 190, 191, 192, 193, 194, 195, 196, 197, 198, 199, 200, 201, 202, 203, 204, 205, 206, 207, 208, 209, 210, 211, 212, 213, 214, 215, 216, 217, 218, 219, 220, 221, 222, 223, 224, 225, 226, 227, 228, 229, 230, 231, 232, 233, 234, 235, 236, 237, 238, 239, 240, 241, 242, 243, 244, 245, 246, 247, 248, 249 or 250 nucleotides or longer.

In some cases, the length of a switch oligo may be at most 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99, 100, 101, 102, 103, 104, 105, 106, 107, 108, 109, 110, 111, 112, 113, 114, 115, 116, 117, 118, 119, 120, 121, 122, 123, 124, 125, 126, 127, 128, 129, 130, 131, 132, 133, 134, 135, 136, 137, 138, 139, 140, 141, 142, 143, 144, 145, 146, 147, 148, 149, 150, 151, 152, 153, 154, 155, 156, 157, 158, 159, 160, 161, 162, 163, 164, 165, 166, 167, 168, 169, 170, 171, 172, 173, 174, 175, 176, 177, 178, 179, 180, 181, 182, 183, 184, 185, 186, 187, 188, 189, 190, 191, 192, 193, 194, 195, 196, 197, 198, 199, 200, 201, 202, 203, 204, 205, 206, 207, 208, 209, 210, 211, 212, 213, 214, 215, 216, 217, 218, 219, 220, 221, 222, 223, 224, 225, 226, 227, 228, 229, 230, 231, 232, 233, 234, 235, 236, 237, 238, 239, 240, 241, 242, 243, 244, 245, 246, 247, 248, 249 or 250 nucleotides.

Once the contents of the cells are released into their respective partitions, the nucleic acids contained therein may be further processed within the partitions. In accordance with the methods and systems described herein, the nucleic acid contents of individual cells are generally provided with unique identifiers such that, upon characterization of those nucleic acids they may be attributed as having been derived from the same cell or cells. The ability to attribute characteristics to individual cells or groups of cells is provided by the assignment of unique identifiers specifically to an individual cell or groups of cells, which is another advantageous aspect of the methods and systems described herein. In particular, unique identifiers, e.g., in the form of nucleic acid barcodes are assigned or associated with individual cells or populations of cells, in order to tag or label the cell's components (and as a result, its characteristics) with the unique identifiers. These unique identifiers are then used to attribute the cell's components and characteristics to an individual cell or group of cells. In some aspects, this is carried out by co-partitioning the individual cells or groups of cells with the unique identifiers. In some aspects, the unique identifiers are provided in the form of oligonucleotides that comprise nucleic acid barcode sequences that may be attached to or otherwise associated with the nucleic acid contents of individual cells, or to other components of the cells, and particularly to fragments of those nucleic acids. The oligonucleotides are partitioned such that as between oligonucleotides in a given partition, the nucleic acid barcode sequences contained therein are the same, but as between different partitions, the oligonucleotides can, and do have differing barcode sequences, or at least represent a large number of different barcode sequences across all of the partitions in a given analysis. In some aspects, only one nucleic acid barcode sequence can be associated with a given partition, although in some cases, two or more different barcode sequences may be present.

The nucleic acid barcode sequences can include from 6 to about 20 or more nucleotides within the sequence of the oligonucleotides. In some cases, the length of a barcode sequence may be 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20 nucleotides or longer. In some cases, the length of a barcode sequence may be at least 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20 nucleotides or longer. In some cases, the length of a barcode sequence may be at most 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20 nucleotides or shorter. These nucleotides may be completely contiguous, i.e., in a single stretch of adjacent nucleotides, or they may be separated into two or more separate subsequences that are separated by 1 or more nucleotides. In some cases, separated barcode subsequences can be from about 4 to about 16 nucleotides in length. In some cases, the barcode subsequence may be 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16 nucleotides or longer. In some cases, the barcode subsequence may be at least 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16 nucleotides or longer. In some cases, the barcode subsequence may be at most 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16 nucleotides or shorter.

The co-partitioned oligonucleotides can also comprise other functional sequences useful in the processing of the nucleic acids from the co-partitioned cells. These sequences include, e.g., targeted or random/universal amplification primer sequences for amplifying the genomic DNA from the individual cells within the partitions while attaching the associated barcode sequences, sequencing primers or primer recognition sites, hybridization or probing sequences, e.g.,

for identification of presence of the sequences or for pulling down barcoded nucleic acids, or any of a number of other potential functional sequences. Again, co-partitioning of oligonucleotides and associated barcodes and other functional sequences, along with sample materials is described in, for example, U.S. Patent Application Nos. 61/940,318, filed Feb. 7, 2014, 61/991,018, filed May 9, 2014, and U.S. patent application Ser. No. 14/316,383, filed Jun. 26, 2014, published as U.S. Patent Publication No. 2014/0378345, as well as U.S. patent application Ser. No. 14/175,935, filed Feb. 7, 2014, published as U.S. Patent Publication No. 2014/0227684, the full disclosures of which are incorporated herein by reference in their entireties for all purposes. As will be appreciated other mechanisms of co-partitioning oligonucleotides may also be employed, including, e.g., coalescence of two or more droplets, where one droplet contains oligonucleotides, or microdispensing of oligonucleotides into partitions, e.g., droplets within microfluidic systems.

Briefly, in one example, beads, microparticles or microcapsules are provided that each include large numbers of the above described oligonucleotides releasably attached to the beads, where all of the oligonucleotides attached to a particular bead will include the same nucleic acid barcode sequence, but where a large number of diverse barcode sequences are represented across the population of beads used. In particularly useful examples, hydrogel beads, e.g., comprising polyacrylamide polymer matrices, are used as a solid support and delivery vehicle for the oligonucleotides into the partitions, as they are capable of carrying large numbers of oligonucleotide molecules, and may be configured to release those oligonucleotides upon exposure to a particular stimulus, as described elsewhere herein. In some cases, the population of beads will provide a diverse barcode sequence library that includes at least 1,000 different barcode sequences, at least 5,000 different barcode sequences, at least 10,000 different barcode sequences, at least at least 50,000 different barcode sequences, at least 100,000 different barcode sequences, at least 1,000,000 different barcode sequences, at least 5,000,000 different barcode sequences, or at least 10,000,000 different barcode sequences. Additionally, each bead can be provided with large numbers of oligonucleotide molecules attached. In particular, the number of molecules of oligonucleotides including the barcode sequence on an individual bead can be at least 1,000 oligonucleotide molecules, at least 5,000 oligonucleotide molecules, at least 10,000 oligonucleotide molecules, at least 50,000 oligonucleotide molecules, at least 100,000 oligonucleotide molecules, at least 500,000 oligonucleotides, at least 1,000,000 oligonucleotide molecules, at least 5,000,000 oligonucleotide molecules, at least 10,000,000 oligonucleotide molecules, at least 50,000,000 oligonucleotide molecules, at least 100,000,000 oligonucleotide molecules, and in some cases at least 1 billion oligonucleotide molecules.

Moreover, when the population of beads is partitioned, the resulting population of partitions can also include a diverse barcode library that includes at least 1,000 different barcode sequences, at least 5,000 different barcode sequences, at least 10,000 different barcode sequences, at least at least 50,000 different barcode sequences, at least 100,000 different barcode sequences, at least 1,000,000 different barcode sequences, at least 5,000,000 different barcode sequences, or at least 10,000,000 different barcode sequences. Additionally, each partition of the population can include at least 1,000 oligonucleotide molecules, at least 5,000 oligonucleotide molecules, at least 10,000 oligonucleotide molecules, at least 50,000 oligonucleotide molecules, at least 100,000 oligonucleotide molecules, at least 500,000 oligonucleotides, at least 1,000,000 oligonucleotide molecules, at least 5,000,000 oligonucleotide molecules, at least 10,000,000 oligonucleotide molecules, at least 50,000,000 oligonucleotide molecules, at least 100,000,000 oligonucleotide molecules, and in some cases at least 1 billion oligonucleotide molecules.

In some cases, it may be desirable to incorporate multiple different barcodes within a given partition, either attached to a single or multiple beads within the partition. For example, in some cases, a mixed, but known barcode sequences set may provide greater assurance of identification in the subsequent processing, e.g., by providing a stronger address or attribution of the barcodes to a given partition, as a duplicate or independent confirmation of the output from a given partition.

The oligonucleotides are releasable from the beads upon the application of a particular stimulus to the beads. In some cases, the stimulus may be a photo-stimulus, e.g., through cleavage of a photo-labile linkage that releases the oligonucleotides. In other cases, a thermal stimulus may be used, where elevation of the temperature of the beads environment will result in cleavage of a linkage or other release of the oligonucleotides form the beads. In still other cases, a chemical stimulus is used that cleaves a linkage of the oligonucleotides to the beads, or otherwise results in release of the oligonucleotides from the beads. Examples of this type of system are described in U.S. patent application Ser. No. 13/966,150, filed Aug. 13, 2013, published as U.S. Patent Publication No. 2014/0378345, as well as U.S. Provisional Patent Application Nos. 61/940,318, filed Feb. 7, 2014, 61/991,018, Filed May 9, 2014, and U.S. patent application Ser. No. 14/316,383, filed Jun. 26, 2014, published as U.S. Patent Publication No. 2014/0378345, the full disclosures of which are hereby incorporated herein by reference n their entireties for all purposes. In one case, such compositions include the polyacrylamide matrices described above for encapsulation of cells, and may be degraded for release of the attached oligonucleotides through exposure to a reducing agent, such as DTT.

In accordance with the methods and systems described herein, the beads including the attached oligonucleotides are co-partitioned with the individual cells, such that a single bead and a single cell are contained within an individual partition. As noted above, while single cell/single bead occupancy is the most desired state, it will be appreciated that multiply occupied partitions (either in terms of cells, beads or both), or unoccupied partitions (either in terms of cells, beads or both) will often be present. An example of a microfluidic channel structure for co-partitioning cells and beads comprising barcode oligonucleotides is schematically illustrated in FIG. **2**. As described elsewhere herein, in some aspects, a substantial percentage of the overall occupied partitions will include both a bead and a cell and, in some cases, some of the partitions that are generated will be unoccupied. In some cases, some of the partitions may have beads and cells that are not partitioned 1:1. In some cases, it may be desirable to provide multiply occupied partitions, e.g., containing two, three, four or more cells and/or beads within a single partition. As shown, channel segments **202**, **204**, **206**, **208** and **210** are provided in fluid communication at channel junction **212**. An aqueous stream comprising the individual cells **214**, is flowed through channel segment **202** toward channel junction **212**. As described above, these cells may be suspended within an aqueous fluid, or may have been pre-encapsulated, prior to the partitioning process.

Concurrently, an aqueous stream comprising the barcode carrying beads **216**, is flowed through channel segment **204** toward channel junction **212**. A non-aqueous partitioning fluid **216** is introduced into channel junction **212** from each of side channels **206** and **208**, and the combined streams are flowed into outlet channel **210**. Within channel junction **212**, the two combined aqueous streams from channel segments **202** and **204** are combined, and partitioned into droplets **218**, that include co-partitioned cells **214** and beads **216**. As noted previously, by controlling the flow characteristics of each of the fluids combining at channel junction **212**, as well as controlling the geometry of the channel junction, one can optimize the combination and partitioning to achieve a desired occupancy level of beads, cells or both, within the partitions **218** that are generated.

In some cases, lysis agents, e.g., cell lysis enzymes, may be introduced into the partition with the bead stream, e.g., flowing through channel segment **204**, such that lysis of the cell only commences at or after the time of partitioning. Additional reagents may also be added to the partition in this configuration, such as endonucleases to fragment the cell's DNA, DNA polymerase enzyme and dNTPs used to amplify the cell's nucleic acid fragments and to attach the barcode oligonucleotides to the amplified fragments. As noted above, in many cases, a chemical stimulus, such as DTT, may be used to release the barcodes from their respective beads into the partition. In such cases, it may be particularly desirable to provide the chemical stimulus along with the cell-containing stream in channel segment **202**, such that release of the barcodes only occurs after the two streams have been combined, e.g., within the partitions **218**. Where the cells are encapsulated, however, introduction of a common chemical stimulus, e.g., that both releases the oligonucleotides form their beads, and releases cells from their microcapsules may generally be provided from a separate additional side channel (not shown) upstream of or connected to channel junction **212**.

As will be appreciated, a number of other reagents may be co-partitioned along with the cells, beads, lysis agents and chemical stimuli, including, for example, protective reagents, like proteinase K, chelators, nucleic acid extension, replication, transcription or amplification reagents such as polymerases, reverse transcriptases, transposases which can be used for transposon based methods (e.g., Nextera), nucleoside triphosphates or NTP analogues, primer sequences and additional cofactors such as divalent metal ions used in such reactions, ligation reaction reagents, such as ligase enzymes and ligation sequences, dyes, labels, or other tagging reagents.

The channel networks, e.g., as described herein, can be fluidly coupled to appropriate fluidic components. For example, the inlet channel segments, e.g., channel segments **202**, **204**, **206** and **208** are fluidly coupled to appropriate sources of the materials they are to deliver to channel junction **212**. For example, channel segment **202** will be fluidly coupled to a source of an aqueous suspension of cells **214** to be analyzed, while channel segment **204** would be fluidly coupled to a source of an aqueous suspension of beads **216**. Channel segments **206** and **208** would then be fluidly connected to one or more sources of the non-aqueous fluid. These sources may include any of a variety of different fluidic components, from simple reservoirs defined in or connected to a body structure of a microfluidic device, to fluid conduits that deliver fluids from off-device sources, manifolds, or the like. Likewise, the outlet channel segment **210** may be fluidly coupled to a receiving vessel or conduit for the partitioned cells. Again, this may be a reservoir defined in the body of a microfluidic device, or it may be a fluidic conduit for delivering the partitioned cells to a subsequent process operation, instrument or component.

FIG. **8** shows images of individual Jurkat cells co-partitioned along with barcode oligonucleotide containing beads in aqueous droplets in an aqueous in oil emulsion. As illustrated, individual cells may be readily co-partitioned with individual beads. As will be appreciated, optimization of individual cell loading may be carried out by a number of methods, including by providing dilutions of cell populations into the microfluidic system in order to achieve the desired cell loading per partition as described elsewhere herein.

In operation, once lysed, the nucleic acid contents of the individual cells are then available for further processing within the partitions, including, e.g., fragmentation, amplification and barcoding, as well as attachment of other functional sequences. As noted above, fragmentation may be accomplished through the co-partitioning of shearing enzymes, such as endonucleases, in order to fragment the nucleic acids into smaller fragments. These endonucleases may include restriction endonucleases, including type II and type IIs restriction endonucleases as well as other nucleic acid cleaving enzymes, such as nicking endonucleases, and the like. In some cases, fragmentation may not be desired, and full length nucleic acids may be retained within the partitions, or in the case of encapsulated cells or cell contents, fragmentation may be carried out prior to partitioning, e.g., through enzymatic methods, e.g., those described herein, or through mechanical methods, e.g., mechanical, acoustic or other shearing.

Once co-partitioned, and the cells are lysed to release their nucleic acids, the oligonucleotides disposed upon the bead may be used to barcode and amplify fragments of those nucleic acids. A particularly elegant process for use of these barcode oligonucleotides in amplifying and barcoding fragments of sample nucleic acids is described in detail in U.S. Provisional Patent Application Nos. 61/940,318, filed Feb. 7, 2014, 61/991,018, Filed May 9, 2014, and U.S. patent application Ser. No. 14/316,383, filed Jun. 26, 2014, published as U.S. Patent Publication No. 2014/0378345, and previously incorporated by reference. Briefly, in one aspect, the oligonucleotides present on the beads that are co-partitioned with the cells, are released from their beads into the partition with the cell's nucleic acids. The oligonucleotides can include, along with the barcode sequence, a primer sequence at its 5'end. This primer sequence may be a random oligonucleotide sequence intended to randomly prime numerous different regions on the cell's nucleic acids, or it may be a specific primer sequence targeted to prime upstream of a specific targeted region of the cell's genome.

Once released, the primer portion of the oligonucleotide can anneal to a complementary region of the cell's nucleic acid. Extension reaction reagents, e.g., DNA polymerase, nucleoside triphosphates, co-factors (e.g., Mg2+ or Mn2+), that are also co-partitioned with the cells and beads, then extend the primer sequence using the cell's nucleic acid as a template, to produce a complementary fragment to the strand of the cell's nucleic acid to which the primer annealed, which complementary fragment includes the oligonucleotide and its associated barcode sequence. Annealing and extension of multiple primers to different portions of the cell's nucleic acids will result in a large pool of overlapping complementary fragments of the nucleic acid, each possessing its own barcode sequence indicative of the partition in which it was created. In some cases, these complementary fragments may themselves be used as a template

primed by the oligonucleotides present in the partition to produce a complement of the complement that again, includes the barcode sequence. In some cases, this replication process is configured such that when the first complement is duplicated, it produces two complementary sequences at or near its termini, to allow formation of a hairpin structure or partial hairpin structure, the reduces the ability of the molecule to be the basis for producing further iterative copies. As described herein, the cell's nucleic acids may include any desired nucleic acids within the cell including, for example, the cell's DNA, e.g., genomic DNA, RNA, e.g., messenger RNA, and the like. For example, in some cases, the methods and systems described herein are used in characterizing expressed mRNA, including, e.g., the presence and quantification of such mRNA, and may include RNA sequencing processes as the characterization process. Alternatively or additionally, the reagents partitioned along with the cells may include reagents for the conversion of mRNA into cDNA, e.g., reverse transcriptase enzymes and reagents, to facilitate sequencing processes where DNA sequencing is employed. In some cases, where the nucleic acids to be characterized comprise RNA, e.g., mRNA, schematic illustration of one example of this is shown in FIG. **3**.

As shown, oligonucleotides that include a barcode sequence are co-partitioned in, e.g., a droplet **302** in an emulsion, along with a sample nucleic acid **304**. As noted elsewhere herein, the oligonucleotides **308** may be provided on a bead **306** that is co-partitioned with the sample nucleic acid **304**, which oligonucleotides are releasable from the bead **306**, as shown in panel A. The oligonucleotides **308** include a barcode sequence **312**, in addition to one or more functional sequences, e.g., sequences **310**, **314** and **316**. For example, oligonucleotide **308** is shown as comprising barcode sequence **312**, as well as sequence **310** that may function as an attachment or immobilization sequence for a given sequencing system, e.g., a P5 sequence used for attachment in flow cells of an Illumina Hiseq® or Miseq® system. As shown, the oligonucleotides also include a primer sequence **316**, which may include a random or targeted N-mer for priming replication of portions of the sample nucleic acid **304**. Also included within oligonucleotide **308** is a sequence **314** which may provide a sequencing priming region, such as a "read1" or R1 priming region, that is used to prime polymerase mediated, template directed sequencing by synthesis reactions in sequencing systems. As will be appreciated, the functional sequences may be selected to be compatible with a variety of different sequencing systems, e.g., 454 Sequencing, Ion Torrent Proton or PGM, Illumina X10, etc., and the requirements thereof. In many cases, the barcode sequence **312**, immobilization sequence **310** and R1 sequence **314** may be common to all of the oligonucleotides attached to a given bead. The primer sequence **316** may vary for random N-mer primers, or may be common to the oligonucleotides on a given bead for certain targeted applications.

As will be appreciated, in some cases, the functional sequences may include primer sequences useful for RNA-seq applications. For example, in some cases, the oligonucleotides may include poly-T primers for priming reverse transcription of RNA for RNA-seq. In still other cases, oligonucleotides in a given partition, e.g., included on an individual bead, may include multiple types of primer sequences in addition to the common barcode sequences, such as both DNA-sequencing and RNA sequencing primers, e.g., poly-T primer sequences included within the oligonucleotides coupled to the bead. In such cases, a single partitioned cell may be both subjected to DNA and RNA sequencing processes.

Based upon the presence of primer sequence **316**, the oligonucleotides can prime the sample nucleic acid as shown in panel B, which allows for extension of the oligonucleotides **308** and **308***a* using polymerase enzymes and other extension reagents also co-partitioned with the bead **306** and sample nucleic acid **304**. As shown in panel C, following extension of the oligonucleotides that, for random N-mer primers, would anneal to multiple different regions of the sample nucleic acid **304**; multiple overlapping complements or fragments of the nucleic acid are created, e.g., fragments **318** and **320**. Although including sequence portions that are complementary to portions of sample nucleic acid, e.g., sequences **322** and **324**, these constructs are generally referred to herein as comprising fragments of the sample nucleic acid **304**, having the attached barcode sequences.

The barcoded nucleic acid fragments may then be subjected to characterization, e.g., through sequence analysis, or they may be further amplified in the process, as shown in panel D. For example, additional oligonucleotides, e.g., oligonucleotide **308***b*, also released from bead **306**, may prime the fragments **318** and **320**. This shown in for fragment **318**. In particular, again, based upon the presence of the random N-mer primer **316***b* in oligonucleotide **308***b* (which in many cases can be different from other random N-mers in a given partition, e.g., primer sequence **316**), the oligonucleotide anneals with the fragment **318**, and is extended to create a complement **326** to at least a portion of fragment **318** which includes sequence **328**, that comprises a duplicate of a portion of the sample nucleic acid sequence. Extension of the oligonucleotide **308***b* continues until it has replicated through the oligonucleotide portion **308** of fragment **318**. As noted elsewhere herein, and as illustrated in panel D, the oligonucleotides may be configured to prompt a stop in the replication by the polymerase at a desired point, e.g., after replicating through sequences **316** and **314** of oligonucleotide **308** that is included within fragment **318**. As described herein, this may be accomplished by different methods, including, for example, the incorporation of different nucleotides and/or nucleotide analogues that are not capable of being processed by the polymerase enzyme used. For example, this may include the inclusion of uracil containing nucleotides within the sequence region **312** to prevent a non-uracil tolerant polymerase to cease replication of that region. As a result a fragment **326** is created that includes the full-length oligonucleotide **308***b* at one end, including the barcode sequence **312**, the attachment sequence **310**, the R1 primer region **314**, and the random N-mer sequence **316***b*. At the other end of the sequence may be included the complement **316'** to the random N-mer of the first oligonucleotide **308**, as well as a complement to all or a portion of the R1 sequence, shown as sequence **314'**. The R1 sequence **314** and its complement **314'** are then able to hybridize together to form a partial hairpin structure **328**. As will be appreciated because the random N-mers differ among different oligonucleotides, these sequences and their complements would not be expected to participate in hairpin formation, e.g., sequence **316'**, which is the complement to random N-mer **316**, would not be expected to be complementary to random N-mer sequence **316***b*. This would not be the case for other applications, e.g., targeted primers, where the N-mers would be common among oligonucleotides within a given partition.

By forming these partial hairpin structures, it allows for the removal of first level duplicates of the sample sequence

from further replication, e.g., preventing iterative copying of copies. The partial hairpin structure also provides a useful structure for subsequent processing of the created fragments, e.g., fragment **326**.

In general, the amplification of the cell's nucleic acids is carried out until the barcoded overlapping fragments within the partition constitute at least 1× coverage of the particular portion or all of the cell's genome, at least 2×, at least 3×, at least 4×, at least 5×, at least 10×, at least 20×, at least 40× or more coverage of the genome or its relevant portion of interest. Once the barcoded fragments are produced, they may be directly sequenced on an appropriate sequencing system, e.g., an Illumina Hiseq®, Miseq® or X10 system, or they may be subjected to additional processing, such as further amplification, attachment of other functional sequences, e.g., second sequencing primers, for reverse reads, sample index sequences, and the like.

All of the fragments from multiple different partitions may then be pooled for sequencing on high throughput sequencers as described herein, where the pooled fragments comprise a large number of fragments derived from the nucleic acids of different cells or small cell populations, but where the fragments from the nucleic acids of a given cell will share the same barcode sequence. In particular, because each fragment is coded as to its partition of origin, and consequently its single cell or small population of cells, the sequence of that fragment may be attributed back to that cell or those cells based upon the presence of the barcode, which will also aid in applying the various sequence fragments from multiple partitions to assembly of individual genomes for different cells. This is schematically illustrated in FIG. **4**. As shown in one example, a first nucleic acid **404** from a first cell **400**, and a second nucleic acid **406** from a second cell **402** are each partitioned along with their own sets of barcode oligonucleotides as described above. The nucleic acids may comprise a chromosome, entire genome or other large nucleic acid from the cells.

Within each partition, each cell's nucleic acids **404** and **406** is then processed to separately provide overlapping set of second fragments of the first fragment(s), e.g., second fragment sets **408** and **410**. This processing also provides the second fragments with a barcode sequence that is the same for each of the second fragments derived from a particular first fragment. As shown, the barcode sequence for second fragment set **408** is denoted by "1" while the barcode sequence for fragment set **410** is denoted by "2". A diverse library of barcodes may be used to differentially barcode large numbers of different fragment sets. However, it is not necessary for every second fragment set from a different first fragment to be barcoded with different barcode sequences. In fact, in many cases, multiple different first fragments may be processed concurrently to include the same barcode sequence. Diverse barcode libraries are described in detail elsewhere herein.

The barcoded fragments, e.g., from fragment sets **408** and **410**, may then be pooled for sequencing using, for example, sequence by synthesis technologies available from Illumina or Ion Torrent division of Thermo-Fisher, Inc. Once sequenced, the sequence reads **412** can be attributed to their respective fragment set, e.g., as shown in aggregated reads **414** and **416**, at least in part based upon the included barcodes, and in some cases, in part based upon the sequence of the fragment itself. The attributed sequence reads for each fragment set are then assembled to provide the assembled sequence for each cell's nucleic acids, e.g., sequences **418** and **420**, which in turn, may be attributed to individual cells, e.g., cells **400** and **402**.

While described in terms of analyzing the genetic material present within cells, the methods and systems described herein may have much broader applicability, including the ability to characterize other aspects of individual cells or cell populations, by allowing for the allocation of reagents to individual cells, and providing for the attributable analysis or characterization of those cells in response to those reagents. These methods and systems are particularly valuable in being able to characterize cells for, e.g., research, diagnostic, pathogen identification, and many other purposes. By way of example, a wide range of different cell surface features, e.g., cell surface proteins like cluster of differentiation or CD proteins, have significant diagnostic relevance in characterization of diseases like cancer.

In one particularly useful application, the methods and systems described herein may be used to characterize cell features, such as cell surface features, e.g., proteins, receptors, etc. In particular, the methods described herein may be used to attach reporter molecules to these cell features, that when partitioned as described above, may be barcoded and analyzed, e.g., using DNA sequencing technologies, to ascertain the presence, and in some cases, relative abundance or quantity of such cell features within an individual cell or population of cells.

In a particular example, a library of potential cell binding ligands, e.g., antibodies, antibody fragments, cell surface receptor binding molecules, or the like, maybe provided associated with a first set of nucleic acid reporter molecules, e.g., where a different reporter oligonucleotide sequence is associated with a specific ligand, and therefore capable of binding to a specific cell surface feature. In some aspects, different members of the library may be characterized by the presence of a different oligonucleotide sequence label, e.g., an antibody to a first type of cell surface protein or receptor would have associated with it a first known reporter oligonucleotide sequence, while an antibody to a second receptor protein would have a different known reporter oligonucleotide sequence associated with it. Prior to co-partitioning, the cells would be incubated with the library of ligands, that may represent antibodies to a broad panel of different cell surface features, e.g., receptors, proteins, etc., and which include their associated reporter oligonucleotides. Unbound ligands are washed from the cells, and the cells are then co-partitioned along with the barcode oligonucleotides described above. As a result, the partitions will include the cell or cells, as well as the bound ligands and their known, associated reporter oligonucleotides.

Without the need for lysing the cells within the partitions, one could then subject the reporter oligonucleotides to the barcoding operations described above for cellular nucleic acids, to produce barcoded, reporter oligonucleotides, where the presence of the reporter oligonucleotides can be indicative of the presence of the particular cell surface feature, and the barcode sequence will allow the attribution of the range of different cell surface features to a given individual cell or population of cells based upon the barcode sequence that was co-partitioned with that cell or population of cells. As a result, one may generate a cell-by-cell profile of the cell surface features within a broader population of cells. This aspect of the methods and systems described herein, is described in greater detail below.

This example is schematically illustrated in FIG. **5**. As shown, a population of cells, represented by cells **502** and **504** are incubated with a library of cell surface associated reagents, e.g., antibodies, cell surface binding proteins, ligands or the like, where each different type of binding group includes an associated nucleic acid reporter molecule

associated with it, shown as ligands and associated reporter molecules **506**, **508**, **510** and **512** (with the reporter molecules being indicated by the differently shaded circles). Where the cell expresses the surface features that are bound by the library, the ligands and their associated reporter molecules can become associated or coupled with the cell surface. Individual cells are then partitioned into separate partitions, e.g., droplets **514** and **516**, along with their associated ligand/reporter molecules, as well as an individual barcode oligonucleotide bead as described elsewhere herein, e.g., beads **522** and **524**, respectively. As with other examples described herein, the barcoded oligonucleotides are released from the beads and used to attach the barcode sequence the reporter molecules present within each partition with a barcode that is common to a given partition, but which varies widely among different partitions. For example, as shown in FIG. **5**, the reporter molecules that associate with cell **502** in partition **514** are barcoded with barcode sequence **518**, while the reporter molecules associated with cell **504** in partition **516** are barcoded with barcode **520**. As a result, one is provided with a library of oligonucleotides that reflects the surface ligands of the cell, as reflected by the reporter molecule, but which is substantially attributable to an individual cell by virtue of a common barcode sequence, allowing a single cell level profiling of the surface characteristics of the cell. As will be appreciated, this process is not limited to cell surface receptors but may be used to identify the presence of a wide variety of specific cell structures, chemistries or other characteristics.

III. Barcoding

Downstream applications, for example DNA sequencing, may rely on the barcodes to identify the origin of a sequence and, for example, to assemble a larger sequence from sequenced fragments. Therefore, it may be desirable to add barcodes to the polynucleotide fragments generated by the methods described herein. Barcodes may be of a variety of different formats, including polynucleotide barcodes. Depending upon the specific application, barcodes may be attached to polynucleotide fragments in a reversible or irreversible manner. Barcodes may also allow for identification and/or quantification of individual polynucleotide fragments during sequencing.

Barcodes may be loaded into partitions so that one or more barcodes are introduced into a particular partition. Each partition may contain a different set of barcodes. This may be accomplished by directly dispensing the barcodes into the partitions, enveloping the barcodes (e.g., in a droplet of an emulsion), or by placing the barcodes within a container that is placed in a partition (e.g., a microcapsule).

For example, a population of microcapsules may be prepared such that a first microcapsule in the population comprises multiple copies of identical barcodes (e.g., polynucleotide bar codes, etc.) and a second microcapsule in the population comprises multiple copies of a barcode that differs from the barcode within the first microcapsule. In some cases, the population of microcapsules may comprise multiple microcapsules (e.g., greater than about 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25, 30, 35, 40, 45, 50, 100, 500, 1000, 5000, 10000, 100000, 1000000, 10000000, 100000000, or 1000000000 microcapsules), each containing multiple copies of a barcode that differs from that contained in the other microcapsules. In some cases, the population may comprise greater than about 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25, 30, 35, 40, 45, 50, 100, 500, 1000, 5000, 10000, 100000, 1000000, 10000000, 100000000, or 1000000000 microcapsules with identical sets of barcodes. In some cases, the population may comprise greater than about 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25, 30, 35, 40, 45, 50, 100, 500, 1000, 5000, 10000, 100000, 1000000, 10000000, 100000000, or 1000000000 microcapsules, wherein the microcapsules each comprise a different combination of barcodes. For example, in some cases the different combinations overlap, such that a first microcapsule may comprise, e.g., barcodes A, B, and C, while a second microcapsule may comprise barcodes A, B, and D. In another example, the different combinations do not overlap, such that a first microcapsule may comprise, e.g., barcodes A, B, and C, while a second microcapsule may comprise barcodes D, E, and F. The use of microcapsules is, of course, optional. All of the combinations described above, and throughout this disclosure, may also be generated by dispending barcodes (and other reagents) directly into partitions (e.g., microwells).

The barcodes may be loaded into the partitions at an expected or predicted ratio of barcodes per species to be barcoded (e.g., polynucleotide fragment, strand of polynucleotide, cell, etc.). In some cases, the barcodes are loaded into partitions such that more than about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 20, 50, 100, 500, 1000, 5000, 10000, or 200000 barcodes are loaded per species. In some cases, the barcodes are loaded in the partitions so that less than about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 20, 50, 100, 500, 1000, 5000, 10000, or 200000 barcodes are loaded per species. In some cases, the average number of barcodes loaded per species is less than, or greater than, about 0.0001, 0.001, 0.01, 0.1, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 20, 50, 100, 500, 1000, 5000, 10000, or 200000 barcodes per species.

When more than one barcode is present per polynucleotide fragment, such barcodes may be copies of the same barcode, or multiple different barcodes. For example, the attachment process may be designed to attach multiple identical barcodes to a single polynucleotide fragment, or multiple different barcodes to the polynucleotide fragment.

A microcapsule may be any of a number of sizes or shapes. In some cases, the shape of the microcapsule may be spherical, ellipsoidal, cylindrical, hexagonal or any other symmetrical or non-symmetrical shape. Any cross-section of the microcapsule may also be of any appropriate shape, include but not limited to: circular, oblong, square, rectangular, hexagonal, or other symmetrical or non-symmetrical shape. In some cases, the microcapsule may be of a specific shape that complements an opening (e.g., surface of a microwell) of the device. For example, the microcapsule may be spherical and the opening of a microwell of the device may be circular.

The microcapsules may be of uniform size (e.g., all of the microcapsules are the same size) or heterogeneous size (e.g., some of the microcapsules are of different sizes). A dimension (e.g., diameter, cross-section, side, etc.) of a microcapsule may be at least about 0.001 μm, 0.01 μm, 0.1 μm, 0.5 μm, 1 μm, 5 μm, 10 μm, 50 μm, 100 μm, 200 μm, 300 μm, 400 μm, 500 μm, 600 μm, 700 μm, 800 μm, 900 μm or 1 nm. In some cases, the microcapsule comprises a microwell that is at most about 0.001 μm, 0.01 μm, 0.1 μm, 0.5 μm, 1 μm, 5 μm, 10 μm, 50 μm, 100 μm, 200 μm, 300 μm, 400 μm, 500 μm, 600 μm, 700 μm, 800 μm, 900 μm or 1 nm.

In some cases, microcapsules are of a size and/or shape so as to allow a limited number of microcapsules to be deposited in individual partitions (e.g., microwells, droplets) of the microcapsule array. Microcapsules may have a specific size and/or shape such that exactly or no more than 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10 capsules fit into an individual microwell; in some cases, on average 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10 capsules fit into an individual microwell. In still

further cases, at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 100, 500, or 1000 capsules fit into an individual microwell.

The methods provided herein may comprise loading a partition (e.g., a microwell, droplet of an emulsion) with the reagents necessary for the attachment of barcodes to polynucleotide fragments. In the case of ligation reactions, reagents including restriction enzymes, ligase enzymes, buffers, adapters, barcodes and the like may be loaded into a partition. In the case barcoding by amplification, reagents including primers, DNA polymerases, DNTPs, buffers, barcodes and the like may be loaded into a partition. As described throughout this disclosure, these reagents may be loaded directly into the partition, or via a container such as a microcapsule. If the reagents are not disposed within a container, they may be loaded into a partition (e.g., a microwell) which may then be sealed with a wax or oil until the reagents are used.

Barcodes may be ligated to a polynucleotide fragment using sticky or blunt ends. Barcoded polynucleotide fragments may also be generated by amplifying a polynucleotide fragment with primers comprising barcodes.

Barcodes may be assembled combinatorially, from smaller components designed to assemble in a modular format. For example, three modules, 1A, 1B, and 1C may be combinatorially assembled to produce barcode 1ABC. Such combinatorial assembly may significantly reduce the cost of synthesizing a plurality of barcodes. For example, a combinatorial system consisting of 3 A modules, 3 B modules, and 3 C modules may generate 3*3*3=27 possible barcode sequences from only 9 modules.

Barcoding and beads of the present disclosure may be performed and used as described in, for example, WO2014/028537 and WO 2014/124338, each of which is entirely incorporated herein by reference.

IV. Applications of Single Cell Analysis

There are a wide variety of different applications of the single cell processing and analysis methods and systems described herein, including analysis of specific individual ells, analysis of different cell types within populations of differing cell types, analysis and characterization of large populations of cells for environmental, human health, epidemiological forensic, or any of a wide variety of different applications.

A particularly valuable application of the single cell analysis processes described herein is in the sequencing and characterization of cancer cells. In particular, conventional analytical techniques, including the ensemble sequencing processes alluded to above, are not highly adept at picking small variations in genomic make-up of cancer cells, particularly where those exist in a sea of normal tissue cells. Further, even as between tumor cells, wide variations can exist and can be masked by the ensemble approaches to sequencing (See, e.g., Patel, et al., Single-cell RNA-seq highlights intratumoral heterogeneity in primary glioblastoma, Science DOI: 10.1126/science.1254257 (Published online Jun. 12, 2014). Cancer cells may be derived from solid tumors, hematological malignancies, cell lines, or obtained as circulating tumor cells, and subjected to the partitioning processes described above. Upon analysis, one can identify individual cell sequences as deriving from a single cell or small group of cells, and distinguish those over normal tissue cell sequences. Further, as described in co-pending U.S. Provisional Patent Application No. 62/017,808, filed Jun. 26, 2014, the full disclosures of which is hereby incorporated herein by reference in its entirety for all purposes, one may also obtain phased sequence information from each cell, allowing clearer characterization of the haplotype variants within a cancer cell. The single cell analysis approach is particularly useful for systems and methods involving low quantities of input nucleic acids, as described in co-pending U.S. Provisional Patent Application No. 62/017,580, filed Jun. 26, 2014, the full disclosures of which is hereby incorporated herein by reference in its entirety for all purposes.

As with cancer cell analysis, the analysis and diagnosis of fetal health or abnormality through the analysis of fetal cells is a difficult task using conventional techniques. In particular, in the absence of relatively invasive procedures, such as amniocentesis obtaining fetal cell samples can employ harvesting those cells from the maternal circulation. As will be appreciated, such circulating fetal cells make up an extremely small fraction of the overall cellular population of that circulation. As a result complex analyses are performed in order to characterize what of the obtained data is likely derived from fetal cells as opposed to maternal cells. By employing the single cell characterization methods and systems described herein, however, one can attribute genetic make up to individual cells, and categorize those cells as maternal or fetal based upon their respective genetic make-up. Further, the genetic sequence of fetal cells may be used to identify any of a number of genetic disorders, including, e.g., aneuploidy such as Down syndrome, Edwards syndrome, and Patau syndrome.

The ability to characterize individual cells from larger diverse populations of cells is also of significant value in both environmental testing as well as in forensic analysis, where samples may, by their nature, be made up of diverse populations of cells and other material that "contaminate" the sample, relative to the cells for which the sample is being tested, e.g., environmental indicator organisms, toxic organisms, and the like for, e.g., environmental and food safety testing, victim and/or perpetrator cells in forensic analysis for sexual assault, and other violent crimes, and the like.

Additional useful applications of the above described single cell sequencing and characterization processes are in the field of neuroscience research and diagnosis. In particular, neural cells can include long interspersed nuclear elements (LINEs), or 'jumping' genes that can move around the genome, which cause each neuron to differ from its neighbor cells. Research has shown that the number of LINEs in human brain exceeds that of other tissues, e.g., heart and liver tissue, with between 80 and 300 unique insertions (See, e.g., Coufal, N. G. et al. *Nature* 460, 1127-1131 (2009)). These differences have been postulated as being related to a person's susceptibility to neuro-logical disorders (see, e.g., Muotri, A. R. et al. *Nature* 468, 443-446 (2010)), or provide the brain with a diversity with which to respond to challenges. As such, the methods described herein may be used in the sequencing and characterization of individual neural cells.

The single cell analysis methods described herein are also useful in the analysis of gene expression, as noted above, both in terms of identification of RNA transcripts and their quantitation. In particular, using the single cell level analysis methods described herein, one can isolate and analyze the RNA transcripts present in individual cells, populations of cells, or subsets of populations of cells. In particular, in some cases, the barcode oligonucleotides may be configured to prime, replicate and consequently yield barcoded fragments of RNA from individual cells. For example, in some cases, the barcode oligonucleotides may include mRNA specific priming sequences, e.g., poly-T primer segments that allow priming and replication of mRNA in a reverse transcription reaction or other targeted priming sequences. Alternatively

or additionally, random RNA priming may be carried out using random N-mer primer segments of the barcode oligonucleotides.

FIG. **6** provides a schematic of one example method for RNA expression analysis in individual cells using the methods described herein. As shown, at operation **602** a cell containing sample is sorted for viable cells, which are quantified and diluted for subsequent partitioning. At operation **604**, the individual cells separately co-partitioned with gel beads bearing the barcoding oligonucleotides as described herein. The cells are lysed and the barcoded oligonucleotides released into the partitions at operation **606**, where they interact with and hybridize to the mRNA at operation **608**, e.g., by virtue of a poly-T primer sequence, which is complementary to the poly-A tail of the mRNA. Using the poly-T barcode oligonucleotide as a priming sequence, a reverse transcription reaction is carried out at operation **610** to synthesize a cDNA transcript of the mRNA that includes the barcode sequence. The barcoded cDNA transcripts are then subjected to additional amplification at operation **612**, e.g., using a PCR process, purification at operation **614**, before they are placed on a nucleic acid sequencing system for determination of the cDNA sequence and its associated barcode sequence(s). In some cases, as shown, operations **602** through **608** can occur while the reagents remain in their original droplet or partition, while operations **612** through **616** can occur in bulk (e.g., outside of the partition). In the case where a partition is a droplet in an emulsion, the emulsion can be broken and the contents of the droplet pooled in order to complete operations **612** through **616**. In some cases, barcode oligonucleotides may be digested with exonucleases after the emulsion is broken. Exonuclease activity can be inhibited by ethylenediaminetetraacetic acid (EDTA) following primer digestion. In some cases, operation **610** may be performed either within the partitions based upon co-partitioning of the reverse transcription mixture, e.g., reverse transcriptase and associated reagents, or it may be performed in bulk.

As noted elsewhere herein, the structure of the barcode oligonucleotides may include a number of sequence elements in addition to the oligonucleotide barcode sequence. One example of a barcode oligonucleotide for use in RNA analysis as described above is shown in FIG. **7**. As shown, the overall oligonucleotide **702** is coupled to a bead **704** by a releasable linkage **706**, such as a disulfide linker. The oligonucleotide may include functional sequences that are used in subsequent processing, such as functional sequence **708**, which may include one or more of a sequencer specific flow cell attachment sequence, e.g., a P5 sequence for Illumina sequencing systems, as well as sequencing primer sequences, e.g., a R1 primer for Illumina sequencing systems. A barcode sequence **710** is included within the structure for use in barcoding the sample RNA. An mRNA specific priming sequence, such as poly-T sequence **712** is also included in the oligonucleotide structure. An anchoring sequence segment **714** may be included to ensure that the poly-T sequence hybridizes at the sequence end of the mRNA. This anchoring sequence can include a random short sequence of nucleotides, e.g., 1-mer, 2-mer, 3-mer or longer sequence, which will ensure that the poly-T segment is more likely to hybridize at the sequence end of the poly-A tail of the mRNA. An additional sequence segment **716** may be provided within the oligonucleotide sequence. In some cases, this additional sequence provides a unique molecular sequence segment, e.g., as a random sequence (e.g., such as a random N-mer sequence) that varies across individual oligonucleotides coupled to a single bead, whereas barcode sequence **710** can be constant among oligonucleotides tethered to an individual bead. This unique sequence serves to provide a unique identifier of the starting mRNA molecule that was captured, in order to allow quantitation of the number of original expressed RNA. As will be appreciated, although shown as a single oligonucleotide tethered to the surface of a bead, individual bead can include tens to hundreds of thousands or even millions of individual oligonucleotide molecules, where, as noted, the barcode segment can be constant or relatively constant for a given bead, but where the variable or unique sequence segment will vary across an individual bead. This unique molecular sequence segment may include from 5 to about 8 or more nucleotides within the sequence of the oligonucleotides. In some cases, the unique molecular sequence segment can be 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19 or 20 nucleotides in length or longer. In some cases, the unique molecular sequence segment can be at least 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19 or 20 nucleotides in length or longer. In some cases, the unique molecular sequence segment can be at most 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19 or 20 nucleotides in length or shorter.

In operation, and with reference to FIGS. **6** and **7**, a cell is co-partitioned along with a barcode bearing bead and lysed while the barcoded oligonucleotides are released from the bead. The poly-T portion of the released barcode oligonucleotide then hybridizes to the poly-A tail of the mRNA. The poly-T segment then primes the reverse transcription of the mRNA to produce a cDNA transcript of the mRNA, but which includes each of the sequence segments **708-716** of the barcode oligonucleotide. Again, because the oligonucleotide **702** includes an anchoring sequence **714**, it will more likely hybridize to and prime reverse transcription at the sequence end of the poly-A tail of the mRNA. Within any given partition, all of the cDNA transcripts of the individual mRNA molecules will include a common barcode sequence segment **710**. However, by including the unique random N-mer sequence, the transcripts made from different mRNA molecules within a given partition will vary at this unique sequence. This provides a quantitation feature that can be identifiable even following any subsequent amplification of the contents of a given partition, e.g., the number of unique segments associated with a common barcode can be indicative of the quantity of mRNA originating from a single partition, and thus, a single cell. As noted above, the transcripts are then amplified, cleaned up and sequenced to identify the sequence of the cDNA transcript of the mRNA, as well as to sequence the barcode segment and the unique sequence segment.

As noted elsewhere herein, while a poly-T primer sequence is described, other targeted or random priming sequences may also be used in priming the reverse transcription reaction. Likewise, although described as releasing the barcoded oligonucleotides into the partition along with the contents of the lysed cells, it will be appreciated that in some cases, the gel bead bound oligonucleotides may be used to hybridize ad capture the mRNA on the solid phase of the gel beads, in order to facilitate the separation of the RNA from other cell contents.

An additional example of a barcode oligonucleotide for use in RNA analysis, including messenger RNA (mRNA, including mRNA obtained from a cell) analysis, is shown in FIG. **9**A. As shown, the overall oligonucleotide **902** can be coupled to a bead **904** by a releasable linkage **906**, such as a disulfide linker. The oligonucleotide may include functional sequences that are used in subsequent processing, such as functional sequence **908**, which may include a

sequencer specific flow cell attachment sequence, e.g., a P5 sequence for Illumina sequencing systems, as well as functional sequence **910**, which may include sequencing primer sequences, e.g., a R1 primer binding site for Illumina sequencing systems. A barcode sequence **912** is included within the structure for use in barcoding the sample RNA. An RNA specific (e.g., mRNA specific) priming sequence, such as poly-T sequence **914** is also included in the oligonucleotide structure. An anchoring sequence segment (not shown) may be included to ensure that the poly-T sequence hybridizes at the sequence end of the mRNA. An additional sequence segment **916** may be provided within the oligonucleotide sequence. This additional sequence can provide a unique molecular sequence segment, e.g., as a random N-mer sequence that varies across individual oligonucleotides coupled to a single bead, whereas barcode sequence **912** can be constant among oligonucleotides tethered to an individual bead. As described elsewhere herein, this unique sequence can serve to provide a unique identifier of the starting mRNA molecule that was captured, in order to allow quantitation of the number of original expressed RNA, e.g., mRNA counting. As will be appreciated, although shown as a single oligonucleotide tethered to the surface of a bead, individual beads can include tens to hundreds of thousands or even millions of individual oligonucleotide molecules, where, as noted, the barcode segment can be constant or relatively constant for a given bead, but where the variable or unique sequence segment will vary across an individual bead.

In an example method of cellular RNA (e.g., mRNA) analysis and in reference to FIG. **9**A, a cell is co-partitioned along with a barcode bearing bead, switch oligo **924**, and other reagents such as reverse transcriptase, a reducing agent and dNTPs into a partition (e.g., a droplet in an emulsion). In operation **950**, the cell is lysed while the barcoded oligonucleotides **902** are released from the bead (e.g., via the action of the reducing agent) and the poly-T segment **914** of the released barcode oligonucleotide then hybridizes to the poly-A tail of mRNA **920** that is released from the cell. Next, in operation **952** the poly-T segment **914** is extended in a reverse transcription reaction using the mRNA as a template to produce a cDNA transcript **922** complementary to the mRNA and also includes each of the sequence segments **908**, **912**, **910**, **916** and **914** of the barcode oligonucleotide. Terminal transferase activity of the reverse transcriptase can add additional bases to the cDNA transcript (e.g., polyC). The switch oligo **924** may then hybridize with the additional bases added to the cDNA transcript and facilitate template switching. A sequence complementary to the switch oligo sequence can then be incorporated into the cDNA transcript **922** via extension of the cDNA transcript **922** using the switch oligo **924** as a template. Within any given partition, all of the cDNA transcripts of the individual mRNA molecules will include a common barcode sequence segment **912**. However, by including the unique random N-mer sequence **916**, the transcripts made from different mRNA molecules within a given partition will vary at this unique sequence. As described elsewhere herein, this provides a quantitation feature that can be identifiable even following any subsequent amplification of the contents of a given partition, e.g., the number of unique segments associated with a common barcode can be indicative of the quantity of mRNA originating from a single partition, and thus, a single cell. Following operation **952**, the cDNA transcript **922** is then amplified with primers **926** (e.g., PCR primers) in operation **954**. Next, the amplified product is then purified (e.g., via solid phase reversible immobilization (SPRI)) in operation **956**. At operation **958**, the amplified product is then sheared, ligated to additional functional sequences, and further amplified (e.g., via PCR). The functional sequences may include a sequencer specific flow cell attachment sequence **930**, e.g., a P7 sequence for Illumina sequencing systems, as well as functional sequence **928**, which may include a sequencing primer binding site, e.g., for a R2 primer for Illumina sequencing systems, as well as functional sequence **932**, which may include a sample index, e.g., an i7 sample index sequence for Illumina sequencing systems. In some cases, operations **950** and **952** can occur in the partition, while operations **954**, **956** and **958** can occur in bulk solution (e.g., in a pooled mixture outside of the partition). In the case where a partition is a droplet in an emulsion, the emulsion can be broken and the contents of the droplet pooled in order to complete operations **954**, **956** and **958**. In some cases, operation **954** may be completed in the partition. In some cases, barcode oligonucleotides may be digested with exonucleases after the emulsion is broken. Exonuclease activity can be inhibited by ethylenediaminetetraacetic acid (EDTA) following primer digestion. Although described in terms of specific sequence references used for certain sequencing systems, e.g., Illumina systems, it will be understood that the reference to these sequences is for illustration purposes only, and the methods described herein may be configured for use with other sequencing systems incorporating specific priming, attachment, index, and other operational sequences used in those systems, e.g., systems available from Ion Torrent, Oxford Nanopore, Genia, Pacific Biosciences, Complete Genomics, and the like.

In an alternative example of a barcode oligonucleotide for use in RNA (e.g., cellular RNA) analysis as shown in FIG. **9**A, functional sequence **908** may be a P7 sequence and functional sequence **910** may be a R2 primer binding site. Moreover, the functional sequence **930** may be a P5 sequence, functional sequence **928** may be a R1 primer binding site, and functional sequence **932** may be an i5 sample index sequence for Illumina sequencing systems. The configuration of the constructs generated by such a barcode oligonucleotide can help minimize (or avoid) sequencing of the poly-T sequence during sequencing.

Shown in FIG. **9**B is another example method for RNA analysis, including cellular mRNA analysis. In this method, the switch oligo **924** is co-partitioned with the individual cell and barcoded bead along with reagents such as reverse transcriptase, a reducing agent and dNTPs into a partition (e.g., a droplet in an emulsion). The switch oligo **924** may be labeled with an additional tag **934**, e.g. biotin. In operation **951**, the cell is lysed while the barcoded oligonucleotides **902** (e.g., as shown in FIG. **9**A) are released from the bead (e.g., via the action of the reducing agent). In some cases, sequence **908** is a P7 sequence and sequence **910** is a R2 primer binding site. In other cases, sequence **908** is a P5 sequence and sequence **910** is a R1 primer binding site. Next, the poly-T segment **914** of the released barcode oligonucleotide hybridizes to the poly-A tail of mRNA **920** that is released from the cell. In operation **953**, the poly-T segment **914** is then extended in a reverse transcription reaction using the mRNA as a template to produce a cDNA transcript **922** complementary to the mRNA and also includes each of the sequence segments **908**, **912**, **910**, **916** and **914** of the barcode oligonucleotide. Terminal transferase activity of the reverse transcriptase can add additional bases to the cDNA transcript (e.g., polyC). The switch oligo **924** may then hybridize with the cDNA transcript and facilitate template switching. A sequence complementary to the

switch oligo sequence can then be incorporated into the cDNA transcript **922** via extension of the cDNA transcript **922** using the switch oligo **924** as a template. Next, an isolation operation **960** can be used to isolate the cDNA transcript **922** from the reagents and oligonucleotides in the partition. The additional tag **934**, e.g. biotin, can be contacted with an interacting tag **936**, e.g., streptavidin, which may be attached to a magnetic bead **938**. At operation **960** the cDNA can be isolated with a pull-down operation (e.g., via magnetic separation, centrifugation) before amplification (e.g., via PCR) in operation **955**, followed by purification (e.g., via solid phase reversible immobilization (SPRI)) in operation **957** and further processing (shearing, ligation of sequences **928**, **932** and **930** and subsequent amplification (e.g., via PCR)) in operation **959**. In some cases where sequence **908** is a P7 sequence and sequence **910** is a R2 primer binding site, sequence **930** is a P5 sequence and sequence **928** is a R1 primer binding site and sequence **932** is an i5 sample index sequence. In some cases where sequence **908** is a P5 sequence and sequence **910** is a R1 primer binding site, sequence **930** is a P7 sequence and sequence **928** is a R2 primer binding site and sequence **932** is an i7 sample index sequence. In some cases, as shown, operations **951** and **953** can occur in the partition, while operations **960**, **955**, **957** and **959** can occur in bulk solution (e.g., in a pooled mixture outside of the partition). In the case where a partition is a droplet in an emulsion, the emulsion can be broken and the contents of the droplet pooled in order to complete operation **960**. The operations **955**, **957**, and **959** can then be carried out following operation **960** after the transcripts are pooled for processing.

Shown in FIG. **9**C is another example method for RNA analysis, including cellular mRNA analysis. In this method, the switch oligo **924** is co-partitioned with the individual cell and barcoded bead along with reagents such as reverse transcriptase, a reducing agent and dNTPs in a partition (e.g., a droplet in an emulsion). In operation **961**, the cell is lysed while the barcoded oligonucleotides **902** (e.g., as shown in FIG. **9**A) are released from the bead (e.g., via the action of the reducing agent). In some cases, sequence **908** is a P7 sequence and sequence **910** is a R2 primer binding site. In other cases, sequence **908** is a P5 sequence and sequence **910** is a R1 primer binding site. Next, the poly-T segment **914** of the released barcode oligonucleotide then hybridizes to the poly-A tail of mRNA **920** that is released from the cell. Next, in operation **963** the poly-T segment **914** is then extended in a reverse transcription reaction using the mRNA as a template to produce a cDNA transcript **922** complementary to the mRNA and also includes each of the sequence segments **908**, **912**, **910**, **916** and **914** of the barcode oligonucleotide. Terminal transferase activity of the reverse transcriptase can add additional bases to the cDNA transcript (e.g., polyC). The switch oligo **924** may then hybridize with the cDNA transcript and facilitate template switching. A sequence complementary to the switch oligo sequence can then be incorporated into the cDNA transcript **922** via extension of the cDNA transcript **922** using the switch oligo **924** as a template. Following operation **961** and operation **963**, mRNA **920** and cDNA transcript **922** are denatured in operation **962**. At operation **964**, a second strand is extended from a primer **940** having an additional tag **942**, e.g. biotin, and hybridized to the cDNA transcript **922**. Also in operation **964**, the biotin labeled second strand can be contacted with an interacting tag **936**, e.g. streptavidin, which may be attached to a magnetic bead **938**. The cDNA can be isolated with a pull-down operation (e.g., via magnetic separation, centrifugation) before amplification (e.g., via polymerase chain reaction (PCR)) in operation **965**, followed by purification (e.g., via solid phase reversible immobilization (SPRI)) in operation **967** and further processing (shearing, ligation of sequences **928**, **932** and **930** and subsequent amplification (e.g., via PCR)) in operation **969**. In some cases where sequence **908** is a P7 sequence and sequence **910** is a R2 primer binding site, sequence **930** is a P5 sequence and sequence **928** is a R1 primer binding site and sequence **932** is an i5 sample index sequence. In some cases where sequence **908** is a P5 sequence and sequence **910** is a R1 primer binding site, sequence **930** is a P7 sequence and sequence **928** is a R2 primer binding site and sequence **932** is an i7 sample index sequence. In some cases, operations **961** and **963** can occur in the partition, while operations **962**, **964**, **965**, **967**, and **969** can occur in bulk (e.g., outside the partition). In the case where a partition is a droplet in an emulsion, the emulsion can be broken and the contents of the droplet pooled in order to complete operations **962**, **964**, **965**, **967** and **969**.

Shown in FIG. **9**D is another example method for RNA analysis, including cellular mRNA analysis. In this method, the switch oligo **924** is co-partitioned with the individual cell and barcoded bead along with reagents such as reverse transcriptase, a reducing agent and dNTPs. In operation **971**, the cell is lysed while the barcoded oligonucleotides **902** (e.g., as shown in FIG. **9**A) are released from the bead (e.g., via the action of the reducing agent). In some cases, sequence **908** is a P7 sequence and sequence **910** is a R2 primer binding site. In other cases, sequence **908** is a P5 sequence and sequence **910** is a R1 primer binding site. Next the poly-T segment **914** of the released barcode oligonucleotide then hybridizes to the poly-A tail of mRNA **920** that is released from the cell. Next in operation **973**, the poly-T segment **914** is then extended in a reverse transcription reaction using the mRNA as a template to produce a cDNA transcript **922** complementary to the mRNA and also includes each of the sequence segments **908**, **912**, **910**, **916** and **914** of the barcode oligonucleotide. Terminal transferase activity of the reverse transcriptase can add additional bases to the cDNA transcript (e.g., polyC). The switch oligo **924** may then hybridize with the cDNA transcript and facilitate template switching. A sequence complementary to the switch oligo sequence can then be incorporated into the cDNA transcript **922** via extension of the cDNA transcript **922** using the switch oligo **924** as a template. In operation **966**, the mRNA **920**, cDNA transcript **922** and switch oligo **924** can be denatured, and the cDNA transcript **922** can be hybridized with a capture oligonucleotide **944** labeled with an additional tag **946**, e.g. biotin. In this operation, the biotin-labeled capture oligonucleotide **944**, which is hybridized to the cDNA transcript, can be contacted with an interacting tag **936**, e.g. streptavidin, which may be attached to a magnetic bead **938**. Following separation from other species (e.g., excess barcoded oligonucleotides) using a pull-down operation (e.g., via magnetic separation, centrifugation), the cDNA transcript can be amplified (e.g., via PCR) with primers **926** at operation **975**, followed by purification (e.g., via solid phase reversible immobilization (SPRI)) in operation **977** and further processing (shearing, ligation of sequences **928**, **932** and **930** and subsequent amplification (e.g., via PCR)) in operation **979**. In some cases where sequence **908** is a P7 sequence and sequence **910** is a R2 primer binding site, sequence **930** is a P5 sequence and sequence **928** is a R1 primer binding site and sequence **932** is an i5 sample index sequence. In other cases where sequence **908** is a P5 sequence and sequence **910** is a R1 primer binding site, sequence **930** is a P7 sequence and

sequence **928** is a R2 primer binding site and sequence **932** is an i7 sample index sequence. In some cases, operations **971** and **973** can occur in the partition, while operations **966**, **975**, **977** (purification), and **979** can occur in bulk (e.g., outside the partition). In the case where a partition is a droplet in an emulsion, the emulsion can be broken and the contents of the droplet pooled in order to complete operations **966**, **975**, **977** and **979**.

Shown in FIG. **9**E is another example method for RNA analysis, including cellular RNA analysis. In this method, an individual cell is co-partitioned along with a barcode bearing bead, a switch oligo **990**, and other reagents such as reverse transcriptase, a reducing agent and dNTPs into a partition (e.g., a droplet in an emulsion). In operation **981**, the cell is lysed while the barcoded oligonucleotides (e.g., **902** as shown in FIG. **9**A) are released from the bead (e.g., via the action of the reducing agent). In some cases, sequence **908** is a P7 sequence and sequence **910** is a R2 primer binding site. In other cases, sequence **908** is a P5 sequence and sequence **910** is a R1 primer binding site. Next, the poly-T segment of the released barcode oligonucleotide then hybridizes to the poly-A tail of mRNA **920** released from the cell. Next at operation **983**, the poly-T segment is then extended in a reverse transcription reaction to produce a cDNA transcript **922** complementary to the mRNA and also includes each of the sequence segments **908**, **912**, **910**, **916** and **914** of the barcode oligonucleotide. Terminal transferase activity of the reverse transcriptase can add additional bases to the cDNA transcript (e.g., polyC). The switch oligo **990** may then hybridize with the cDNA transcript and facilitate template switching. A sequence complementary to the switch oligo sequence and including a T7 promoter sequence, can be incorporated into the cDNA transcript **922**. At operation **968**, a second strand is synthesized and at operation **970** the T7 promoter sequence can be used by T7 polymerase to produce RNA transcripts in in vitro transcription. At operation **985** the RNA transcripts can be purified (e.g., via solid phase reversible immobilization (SPRI)), reverse transcribed to form DNA transcripts, and a second strand can be synthesized for each of the DNA transcripts. In some cases, prior to purification, the RNA transcripts can be contacted with a DNase (e.g., DNAase I) to break down residual DNA. At operation **987** the DNA transcripts are then fragmented and ligated to additional functional sequences, such as sequences **928**, **932** and **930** and, in some cases, further amplified (e.g., via PCR). In some cases where sequence **908** is a P7 sequence and sequence **910** is a R2 primer binding site, sequence **930** is a P5 sequence and sequence **928** is a R1 primer binding site and sequence **932** is an i5 sample index sequence. In some cases where sequence **908** is a P5 sequence and sequence **910** is a R1 primer binding site, sequence **930** is a P7 sequence and sequence **928** is a R2 primer binding site and sequence **932** is an i7 sample index sequence. In some cases, prior to removing a portion of the DNA transcripts, the DNA transcripts can be contacted with an RNase to break down residual RNA. In some cases, operations **981** and **983** can occur in the partition, while operations **968**, **970**, **985** and **987** can occur in bulk (e.g., outside the partition). In the case where a partition is a droplet in an emulsion, the emulsion can be broken and the contents of the droplet pooled in order to complete operations **968**, **970**, **985** and **987**.

Another example of a barcode oligonucleotide for use in RNA analysis, including messenger RNA (mRNA, including mRNA obtained from a cell) analysis is shown in FIG. **10**. As shown, the overall oligonucleotide **1002** is coupled to a bead **1004** by a releasable linkage **1006**, such as a disulfide linker. The oligonucleotide may include functional sequences that are used in subsequent processing, such as functional sequence **1008**, which may include a sequencer specific flow cell attachment sequence, e.g., a P7 sequence, as well as functional sequence **1010**, which may include sequencing primer sequences, e.g., a R2 primer binding site. A barcode sequence **1012** is included within the structure for use in barcoding the sample RNA. An RNA specific (e.g., mRNA specific) priming sequence, such as poly-T sequence **1014** may be included in the oligonucleotide structure. An anchoring sequence segment (not shown) may be included to ensure that the poly-T sequence hybridizes at the sequence end of the mRNA. An additional sequence segment **1016** may be provided within the oligonucleotide sequence. This additional sequence can provide a unique molecular sequence segment, as described elsewhere herein. An additional functional sequence **1020** may be included for in vitro transcription, e.g., a T7 RNA polymerase promoter sequence. As will be appreciated, although shown as a single oligonucleotide tethered to the surface of a bead, individual beads can include tens to hundreds of thousands or even millions of individual oligonucleotide molecules, where, as noted, the barcode segment can be constant or relatively constant for a given bead, but where the variable or unique sequence segment will vary across an individual bead.

In an example method of cellular RNA analysis and in reference to FIG. **10**, a cell is co-partitioned along with a barcode bearing bead, and other reagents such as reverse transcriptase, reducing agent and dNTPs into a partition (e.g., a droplet in an emulsion). In operation **1050**, the cell is lysed while the barcoded oligonucleotides **1002** are released (e.g., via the action of the reducing agent) from the bead, and the poly-T segment **1014** of the released barcode oligonucleotide then hybridizes to the poly-A tail of mRNA **1020**. Next at operation **1052**, the poly-T segment is then extended in a reverse transcription reaction using the mRNA as template to produce a cDNA transcript **1022** of the mRNA and also includes each of the sequence segments **1020**, **1008**, **1012**, **1010**, **1016**, and **1014** of the barcode oligonucleotide. Within any given partition, all of the cDNA transcripts of the individual mRNA molecules will include a common barcode sequence segment **1012**. However, by including the unique random N-mer sequence, the transcripts made from different mRNA molecules within a given partition will vary at this unique sequence. As described elsewhere herein, this provides a quantitation feature that can be identifiable even following any subsequent amplification of the contents of a given partition, e.g., the number of unique segments associated with a common barcode can be indicative of the quantity of mRNA originating from a single partition, and thus, a single cell. At operation **1054** a second strand is synthesized and at operation **1056** the T7 promoter sequence can be used by T7 polymerase to produce RNA transcripts in in vitro transcription. At operation **1058** the transcripts are fragmented (e.g., sheared), ligated to additional functional sequences, and reverse transcribed. The functional sequences may include a sequencer specific flow cell attachment sequence **1030**, e.g., a P5 sequence, as well as functional sequence **1028**, which may include sequencing primers, e.g., a R1 primer binding sequence, as well as functional sequence **1032**, which may include a sample index, e.g., an i5 sample index sequence. At operation **1060** the RNA transcripts can be reverse transcribed to DNA, the DNA amplified (e.g., via PCR), and sequenced to identify the sequence of the cDNA transcript of the mRNA, as well as to sequence the barcode segment and the unique sequence segment. In some cases, operations **1050** and **1052** can occur

in the partition, while operations **1054**, **1056**, **1058** and **1060** can occur in bulk (e.g., outside the partition). In the case where a partition is a droplet in an emulsion, the emulsion can be broken and the contents of the droplet pooled in order to complete operations **1054**, **1056**, **1058** and **1060**.

In an alternative example of a barcode oligonucleotide for use in RNA (e.g., cellular RNA) analysis as shown in FIG. **10**, functional sequence **1008** may be a P5 sequence and functional sequence **1010** may be a R1 primer binding site. Moreover, the functional sequence **1030** may be a P7 sequence, functional sequence **1028** may be a R2 primer binding site, and functional sequence **1032** may be an i7 sample index sequence.

An additional example of a barcode oligonucleotide for use in RNA analysis, including messenger RNA (mRNA, including mRNA obtained from a cell) analysis is shown in FIG. **11**. As shown, the overall oligonucleotide **1102** is coupled to a bead **1104** by a releasable linkage **1106**, such as a disulfide linker. The oligonucleotide may include functional sequences that are used in subsequent processing, such as functional sequence **1108**, which may include a sequencer specific flow cell attachment sequence, e.g., a P5 sequence, as well as functional sequence **1110**, which may include sequencing primer sequences, e.g., a R1 primer binding site. In some cases, sequence **1108** is a P7 sequence and sequence **1110** is a R2 primer binding site. A barcode sequence **1112** is included within the structure for use in barcoding the sample RNA. An additional sequence segment **1116** may be provided within the oligonucleotide sequence. In some cases, this additional sequence can provide a unique molecular sequence segment, as described elsewhere herein. An additional sequence **1114** may be included to facilitate template switching, e.g., polyG. As will be appreciated, although shown as a single oligonucleotide tethered to the surface of a bead, individual beads can include tens to hundreds of thousands or even millions of individual oligonucleotide molecules, where, as noted, the barcode segment can be constant or relatively constant for a given bead, but where the variable or unique sequence segment will vary across an individual bead.

In an example method of cellular mRNA analysis and in reference to FIG. **11**, a cell is co-partitioned along with a barcode bearing bead, poly-T sequence, and other reagents such as reverse transcriptase, a reducing agent and dNTPs into a partition (e.g., a droplet in an emulsion). In operation **1150**, the cell is lysed while the barcoded oligonucleotides are released from the bead (e.g., via the action of the reducing agent) and the poly-T sequence hybridizes to the poly-A tail of mRNA **1120** released from the cell. Next, in operation **1152**, the poly-T sequence is then extended in a reverse transcription reaction using the mRNA as a template to produce a cDNA transcript **1122** complementary to the mRNA. Terminal transferase activity of the reverse transcriptase can add additional bases to the cDNA transcript (e.g., polyC). The additional bases added to the cDNA transcript, e.g., polyC, can then to hybridize with **1114** of the barcoded oligonucleotide. This can facilitate template switching and a sequence complementary to the barcode oligonucleotide can be incorporated into the cDNA transcript. The transcripts can be further processed (e.g., amplified, portions removed, additional sequences added, etc.) and characterized as described elsewhere herein, e.g., by sequencing. The configuration of the constructs generated by such a method can help minimize (or avoid) sequencing of the poly-T sequence during sequencing.

An additional example of a barcode oligonucleotide for use in RNA analysis, including cellular RNA analysis is shown in FIG. **12**A. As shown, the overall oligonucleotide **1202** is coupled to a bead **1204** by a releasable linkage **1206**, such as a disulfide linker. The oligonucleotide may include functional sequences that are used in subsequent processing, such as functional sequence **1208**, which may include a sequencer specific flow cell attachment sequence, e.g., a P5 sequence, as well as functional sequence **1210**, which may include sequencing primer sequences, e.g., a R1 primer binding site. In some cases, sequence **1208** is a P7 sequence and sequence **1210** is a R2 primer binding site. A barcode sequence **1212** is included within the structure for use in barcoding the sample RNA. An additional sequence segment **1216** may be provided within the oligonucleotide sequence. In some cases, this additional sequence can provide a unique molecular sequence segment, as described elsewhere herein. As will be appreciated, although shown as a single oligonucleotide tethered to the surface of a bead, individual beads can include tens to hundreds of thousands or even millions of individual oligonucleotide molecules, where, as noted, the barcode segment can be constant or relatively constant for a given bead, but where the variable or unique sequence segment will vary across an individual bead. In an example method of cellular RNA analysis using this barcode, a cell is co-partitioned along with a barcode bearing bead and other reagents such as RNA ligase and a reducing agent into a partition (e.g. a droplet in an emulsion). The cell is lysed while the barcoded oligonucleotides are released (e.g., via the action of the reducing agent) from the bead. The barcoded oligonucleotides can then be ligated to the 5' end of mRNA transcripts while in the partitions by RNA ligase. Subsequent operations may include purification (e.g., via solid phase reversible immobilization (SPRI)) and further processing (shearing, ligation of functional sequences, and subsequent amplification (e.g., via PCR)), and these operations may occur in bulk (e.g., outside the partition). In the case where a partition is a droplet in an emulsion, the emulsion can be broken and the contents of the droplet pooled for the additional operations.

An additional example of a barcode oligonucleotide for use in RNA analysis, including cellular RNA analysis is shown in FIG. **12**B. As shown, the overall oligonucleotide **1222** is coupled to a bead **1224** by a releasable linkage **1226**, such as a disulfide linker. The oligonucleotide may include functional sequences that are used in subsequent processing, such as functional sequence **1228**, which may include a sequencer specific flow cell attachment sequence, e.g., a P5 sequence, as well as functional sequence **1230**, which may include sequencing primer sequences, e.g., a R1 primer binding site. In some cases, sequence **1228** is a P7 sequence and sequence **1230** is a R2 primer binding site. A barcode sequence **1232** is included within the structure for use in barcoding the sample RNA. A priming sequence **1234** (e.g., a random priming sequence) can also be included in the oligonucleotide structure, e.g., a random hexamer. An additional sequence segment **1236** may be provided within the oligonucleotide sequence. In some cases, this additional sequence provides a unique molecular sequence segment, as described elsewhere herein. As will be appreciated, although shown as a single oligonucleotide tethered to the surface of a bead, individual beads can include tens to hundreds of thousands or even millions of individual oligonucleotide molecules, where, as noted, the barcode segment can be constant or relatively constant for a given bead, but where the variable or unique sequence segment will vary across an individual bead. In an example method of cellular mRNA analysis using the barcode oligonucleotide of FIG. **12**B, a cell is co-partitioned along with a barcode bearing bead and

additional reagents such as reverse transcriptase, a reducing agent and dNTPs into a partition (e.g., a droplet in an emulsion). The cell is lysed while the barcoded oligonucleotides are released from the bead (e.g., via the action of the reducing agent). In some cases, sequence **1228** is a P7 sequence and sequence **1230** is a R2 primer binding site. In other cases, sequence **1228** is a P5 sequence and sequence **1230** is a R1 primer binding site. The priming sequence **1234** of random hexamers can randomly hybridize cellular mRNA. The random hexamer sequence can then be extended in a reverse transcription reaction using mRNA from the cell as a template to produce a cDNA transcript complementary to the mRNA and also includes each of the sequence segments **1228**, **1232**, **1230**, **1236**, and **1234** of the barcode oligonucleotide. Subsequent operations may include purification (e.g., via solid phase reversible immobilization (SPRI)), further processing (shearing, ligation of functional sequences, and subsequent amplification (e.g., via PCR)), and these operations may occur in bulk (e.g., outside the partition). In the case where a partition is a droplet in an emulsion, the emulsion can be broken and the contents of the droplet pooled for additional operations. Additional reagents that may be co-partitioned along with the barcode bearing bead may include oligonucleotides to block ribosomal RNA (rRNA) and nucleases to digest genomic DNA and cDNA from cells. Alternatively, rRNA removal agents may be applied during additional processing operations. The configuration of the constructs generated by such a method can help minimize (or avoid) sequencing of the poly-T sequence during sequencing.

The single cell analysis methods described herein may also be useful in the analysis of the whole transcriptome. Referring back to the barcode of FIG. **12**B, the priming sequence **1234** may be a random N-mer. In some cases, sequence **1228** is a P7 sequence and sequence **1230** is a R2 primer binding site. In other cases, sequence **1228** is a P5 sequence and sequence **1230** is a R1 primer binding site. In an example method of whole transcriptome analysis using this barcode, the individual cell is co-partitioned along with a barcode bearing bead, poly-T sequence, and other reagents such as reverse transcriptase, polymerase, a reducing agent and dNTPs into a partition (e.g., droplet in an emulsion). In an operation of this method, the cell is lysed while the barcoded oligonucleotides are released from the bead (e.g., via the action of the reducing agent) and the poly-T sequence hybridizes to the poly-A tail of cellular mRNA. In a reverse transcription reaction using the mRNA as template, cDNA transcripts of cellular mRNA can be produced. The RNA can then be degraded with an RNase. The priming sequence **1234** in the barcoded oligonucleotide can then randomly hybridize to the cDNA transcripts. The oligonucleotides can be extended using polymerase enzymes and other extension reagents co-partitioned with the bead and cell similar to as shown in FIG. **3** to generate amplification products (e.g., barcoded fragments), similar to the example amplification product shown in FIG. **3** (panel F). The barcoded nucleic acid fragments may, in some cases subjected to further processing (e.g., amplification, addition of additional sequences, clean up processes, etc. as described elsewhere herein) characterized, e.g., through sequence analysis. In this operation, sequencing signals can come from full length RNA.

Although operations with various barcode designs have been discussed individually, individual beads can include barcode oligonucleotides of various designs for simultaneous use.

In addition to characterizing individual cells or cell subpopulations from larger populations, the processes and systems described herein may also be used to characterize individual cells as a way to provide an overall profile of a cellular, or other organismal population. A variety of applications require the evaluation of the presence and quantification of different cell or organism types within a population of cells, including, for example, microbiome analysis and characterization, environmental testing, food safety testing, epidemiological analysis, e.g., in tracing contamination or the like. In particular, the analysis processes described above may be used to individually characterize, sequence and/or identify large numbers of individual cells within a population. This characterization may then be used to assemble an overall profile of the originating population, which can provide important prognostic and diagnostic information.

For example, shifts in human microbiomes, including, e.g., gut, buccal, epidermal microbiomes, etc., have been identified as being both diagnostic and prognostic of different conditions or general states of health. Using the single cell analysis methods and systems described herein, one can again, characterize, sequence and identify individual cells in an overall population, and identify shifts within that population that may be indicative of diagnostic ally relevant factors. By way of example, sequencing of bacterial 16S ribosomal RNA genes has been used as a highly accurate method for taxonomic classification of bacteria. Using the targeted amplification and sequencing processes described above can provide identification of individual cells within a population of cells. One may further quantify the numbers of different cells within a population to identify current states or shifts in states over time. See, e.g., Morgan et al, PLoS Comput. Biol., Ch. 12, December 2012, 8(12):e1002808, and Ram et al., Syst. Biol. Reprod. Med., June 2011, 57(3):162-170, each of which is incorporated herein by reference in its entirety for all purposes. Likewise, identification and diagnosis of infection or potential infection may also benefit from the single cell analyses described herein, e.g., to identify microbial species present in large mixes of other cells or other biological material, cells and/or nucleic acids, including the environments described above, as well as any other diagnostically relevant environments, e.g., cerebrospinal fluid, blood, fecal or intestinal samples, or the like.

The foregoing analyses may also be particularly useful in the characterization of potential drug resistance of different cells, e.g., cancer cells, bacterial pathogens, etc., through the analysis of distribution and profiling of different resistance markers/mutations across cell populations in a given sample. Additionally, characterization of shifts in these markers/mutations across populations of cells over time can provide valuable insight into the progression, alteration, prevention, and treatment of a variety of diseases characterized by such drug resistance issues.

Although described in terms of cells, it will be appreciated that any of a variety of individual biological organisms, or components of organisms are encompassed within this description, including, for example, cells, viruses, organelles, cellular inclusions, vesicles, or the like. Additionally, where referring to cells, it will be appreciated that such reference includes any type of cell, including without limitation prokaryotic cells, eukaryotic cells, bacterial, fungal, plant, mammalian, or other animal cell types, mycoplasmas, normal tissue cells, tumor cells, or any other cell type, whether derived from single cell or multicellular organisms.

Similarly, analysis of different environmental samples to profile the microbial organisms, viruses, or other biological

contaminants that are present within such samples, can provide important information about disease epidemiology, and potentially aid in forecasting disease outbreaks, epidemics an pandemics.

As described above, the methods, systems and compositions described herein may also be used for analysis and characterization of other aspects of individual cells or populations of cells. In one example process, a sample is provided that contains cells that are to be analyzed and characterized as to their cell surface proteins. Also provided is a library of antibodies, antibody fragments, or other molecules having a binding affinity to the cell surface proteins or antigens (or other cell features) for which the cell is to be characterized (also referred to herein as cell surface feature binding groups). For ease of discussion, these affinity groups are referred to herein as binding groups. The binding groups can include a reporter molecule that is indicative of the cell surface feature to which the binding group binds. In particular, a binding group type that is specific to one type of cell surface feature will comprise a first reporter molecule, while a binding group type that is specific to a different cell surface feature will have a different reporter molecule associated with it. In some aspects, these reporter molecules will comprise oligonucleotide sequences. Oligonucleotide based reporter molecules provide advantages of being able to generate significant diversity in terms of sequence, while also being readily attachable to most biomolecules, e.g., antibodies, etc., as well as being readily detected, e.g., using sequencing or array technologies. In the example process, the binding groups include oligonucleotides attached to them. Thus, a first binding group type, e.g., antibodies to a first type of cell surface feature, will have associated with it a reporter oligonucleotide that has a first nucleotide sequence. Different binding group types, e.g., antibodies having binding affinity for other, different cell surface features, will have associated therewith reporter oligonucleotides that comprise different nucleotide sequences, e.g., having a partially or completely different nucleotide sequence. In some cases, for each type of cell surface feature binding group, e.g., antibody or antibody fragment, the reporter oligonucleotide sequence may be known and readily identifiable as being associated with the known cell surface feature binding group. These oligonucleotides may be directly coupled to the binding group, or they may be attached to a bead, molecular lattice, e.g., a linear, globular, cross-slinked, or other polymer, or other framework that is attached or otherwise associated with the binding group, which allows attachment of multiple reporter oligonucleotides to a single binding group.

In the case of multiple reporter molecules coupled to a single binding group, such reporter molecules can comprise the same sequence, or a particular binding group will include a known set of reporter oligonucleotide sequences. As between different binding groups, e.g., specific for different cell surface features, the reporter molecules can be different and attributable to the particular binding group.

Attachment of the reporter groups to the binding groups may be achieved through any of a variety of direct or indirect, covalent or non-covalent associations or attachments. For example, in the case of oligonucleotide reporter groups associated with antibody based binding groups, such oligonucleotides may be covalently attached to a portion of an antibody or antibody fragment using chemical conjugation techniques (e.g., Lightning-Link® antibody labeling kits available from Innova Biosciences), as well as other non-covalent attachment mechanisms, e.g., using biotinylated antibodies and oligonucleotides (or beads that include one or more biotinylated linker, coupled to oligonucleotides) with an avidin or streptavidin linker. Antibody and oligonucleotide biotinylation techniques are available (See, e.g., Fang, et al., *Fluoride-Cleavable Biotinylation Phosphoramidite for 5'-end-Labeling and Affinity Purification of Synthetic Oligonucleotides*, Nucleic Acids Res. Jan. 15, 2003; 31(2):708-715, DNA 3' End Biotinylation Kit, available from Thermo Scientific, the full disclosures of which are incorporated herein by reference in their entirety for all purposes). Likewise, protein and peptide biotinylation techniques have been developed and are readily available (See, e.g., U.S. Pat. No. 6,265,552, the full disclosures of which are incorporated herein by reference in their entirety for all purposes).

The reporter oligonucleotides may be provided having any of a range of different lengths, depending upon the diversity of reporter molecules desired or a given analysis, the sequence detection scheme employed, and the like. In some cases, these reporter sequences can be greater than about 5 nucleotides in length, greater than about 10 nucleotides in length, greater than about 20, 30, 40, 50, 60, 70, 80, 90, 100, 120, 150 or even 200 nucleotides in length. In some cases, these reporter nucleotides may be less than about 250 nucleotides in length, less than about 200, 180, 150, 120 100, 90, 80, 70, 60, 50, 40, or even 30 nucleotides in length. In many cases, the reporter oligonucleotides may be selected to provide barcoded products that are already sized, and otherwise configured to be analyzed on a sequencing system. For example, these sequences may be provided at a length that ideally creates sequenceable products of a desired length for particular sequencing systems. Likewise, these reporter oligonucleotides may include additional sequence elements, in addition to the reporter sequence, such as sequencer attachment sequences, sequencing primer sequences, amplification primer sequences, or the complements to any of these.

In operation, a cell-containing sample is incubated with the binding molecules and their associated reporter oligonucleotides, for any of the cell surface features desired to be analyzed. Following incubation, the cells are washed to remove unbound binding groups. Following washing, the cells are partitioned into separate partitions, e.g., droplets, along with the barcode carrying beads described above, where each partition includes a limited number of cells, e.g., in some cases, a single cell. Upon releasing the barcodes from the beads, they will prime the amplification and barcoding of the reporter oligonucleotides. As noted above, the barcoded replicates of the reporter molecules may additionally include functional sequences, such as primer sequences, attachment sequences or the like.

The barcoded reporter oligonucleotides are then subjected to sequence analysis to identify which reporter oligonucleotides bound to the cells within the partitions. Further, by also sequencing the associated barcode sequence, one can identify that a given cell surface feature likely came from the same cell as other, different cell surface features, whose reporter sequences include the same barcode sequence, i.e., they were derived from the same partition.

Based upon the reporter molecules that emanate from an individual partition based upon the presence of the barcode sequence, one may then create a cell surface profile of individual cells from a population of cells. Profiles of individual cells or populations of cells may be compared to profiles from other cells, e.g., 'normal' cells, to identify variations in cell surface features, which may provide diagnostically relevant information. In particular, these profiles may be particularly useful in the diagnosis of a variety of

disorders that are characterized by variations in cell surface receptors, such as cancer and other disorders.

Methods of the disclosure may be applicable to processing samples for the detection of changes in gene expression. A sample may comprise a cell, mRNA, or cDNA reverse transcribed from mRNA. The sample may be a pooled sample, comprising extracts from several different cells or tissues, or a sample comprising extracts from a single cell or tissue. Methods of the invention may be used to fragment and barcode the polynucleotides of the cell.

Cells may be placed directly into a partition (e.g., a microwell) and lysed. After lysis, the sequencing. Polynucleotides may also be extracted from cells prior to introducing them into a partition used in a method of the invention. Reverse transcription of mRNA may be performed in a partition described herein, or outside of such a partition. Sequencing cDNA may provide an indication of the abundance of a particular transcript in a particular cell over time, or after exposure to a particular condition.

V. Devices and Systems

Also provided herein are the microfluidic devices used for partitioning the cells as described above. Such microfluidic devices can comprise channel networks for carrying out the partitioning process like those set forth in FIGS. **1** and **2**. Examples of particularly useful microfluidic devices are described in U.S. Provisional Patent Application No. 61/977,804, filed Apr. 4, 2014, and incorporated herein by reference in its entirety for all purposes. Briefly, these microfluidic devices can comprise channel networks, such as those described herein, for partitioning cells into separate partitions, and co-partitioning such cells with oligonucleotide barcode library members, e.g., disposed on beads. These channel networks can be disposed within a solid body, e.g., a glass, semiconductor or polymer body structure in which the channels are defined, where those channels communicate at their termini with reservoirs for receiving the various input fluids, and for the ultimate deposition of the partitioned cells, etc., from the output of the channel networks. By way of example, and with reference to FIG. **2**, a reservoir fluidly coupled to channel **202** may be provided with an aqueous suspension of cells **214**, while a reservoir coupled to channel **204** may be provided with an aqueous suspension of beads **216** carrying the oligonucleotides. Channel segments **206** and **208** may be provided with a non-aqueous solution, e.g., an oil, into which the aqueous fluids are partitioned as droplets at the channel junction **212**. Finally, an outlet reservoir may be fluidly coupled to channel **210** into which the partitioned cells and beads can be delivered and from which they may be harvested. As will be appreciated, while described as reservoirs, it will be appreciated that the channel segments may be coupled to any of a variety of different fluid sources or receiving components, including tubing, manifolds, or fluidic components of other systems.

Also provided are systems that control flow of these fluids through the channel networks e.g., through applied pressure differentials, centrifugal force, electrokinetic pumping, capillary or gravity flow, or the like.

VI. Kits

Also provided herein are kits for analyzing individual cells or small populations of cells. The kits may include one, two, three, four, five or more, up to all of partitioning fluids, including both aqueous buffers and non-aqueous partitioning fluids or oils, nucleic acid barcode libraries that are releasably associated with beads, as described herein, microfluidic devices, reagents for disrupting cells amplifying nucleic acids, and providing additional functional sequences on fragments of cellular nucleic acids or replicates thereof, as well as instructions for using any of the foregoing in the methods described herein.

VII. Computer Control Systems

The present disclosure provides computer control systems that are programmed to implement methods of the disclosure. FIG. **17** shows a computer system **1701** that is programmed or otherwise configured to implement methods of the disclosure including nucleic acid sequencing methods, interpretation of nucleic acid sequencing data and analysis of cellular nucleic acids, such as RNA (e.g., mRNA), and characterization of cells from sequencing data. The computer system **1701** can be an electronic device of a user or a computer system that is remotely located with respect to the electronic device. The electronic device can be a mobile electronic device.

The computer system **1701** includes a central processing unit (CPU, also "processor" and "computer processor" herein) **1705**, which can be a single core or multi core processor, or a plurality of processors for parallel processing. The computer system **1701** also includes memory or memory location **1710** (e.g., random-access memory, read-only memory, flash memory), electronic storage unit **1715** (e.g., hard disk), communication interface **1720** (e.g., network adapter) for communicating with one or more other systems, and peripheral devices **1725**, such as cache, other memory, data storage and/or electronic display adapters. The memory **1710**, storage unit **1715**, interface **1720** and peripheral devices **1725** are in communication with the CPU **1705** through a communication bus (solid lines), such as a motherboard. The storage unit **1715** can be a data storage unit (or data repository) for storing data. The computer system **1701** can be operatively coupled to a computer network ("network") **1730** with the aid of the communication interface **1720**. The network **1730** can be the Internet, an internet and/or extranet, or an intranet and/or extranet that is in communication with the Internet. The network **1730** in some cases is a telecommunication and/or data network. The network **1730** can include one or more computer servers, which can enable distributed computing, such as cloud computing. The network **1730**, in some cases with the aid of the computer system **1701**, can implement a peer-to-peer network, which may enable devices coupled to the computer system **1701** to behave as a client or a server.

The CPU **1705** can execute a sequence of machine-readable instructions, which can be embodied in a program or software. The instructions may be stored in a memory location, such as the memory **1710**. The instructions can be directed to the CPU **1705**, which can subsequently program or otherwise configure the CPU **1705** to implement methods of the present disclosure. Examples of operations performed by the CPU **1705** can include fetch, decode, execute, and writeback.

The CPU **1705** can be part of a circuit, such as an integrated circuit. One or more other components of the system **1701** can be included in the circuit. In some cases, the circuit is an application specific integrated circuit (ASIC).

The storage unit **1715** can store files, such as drivers, libraries and saved programs. The storage unit **1715** can store user data, e.g., user preferences and user programs. The computer system **1701** in some cases can include one or more additional data storage units that are external to the computer system **1701**, such as located on a remote server that is in communication with the computer system **1701** through an intranet or the Internet.

The computer system **1701** can communicate with one or more remote computer systems through the network **1730**. For instance, the computer system **1701** can communicate with a remote computer system of a user. Examples of remote computer systems include personal computers (e.g., portable PC), slate or tablet PC's (e.g., Apple® iPad, Samsung® Galaxy Tab), telephones, Smart phones (e.g., Apple® iPhone, Android-enabled device, Blackberry®), or personal digital assistants. The user can access the computer system **1701** via the network **1730**.

Methods as described herein can be implemented by way of machine (e.g., computer processor) executable code stored on an electronic storage location of the computer system **1701**, such as, for example, on the memory **1710** or electronic storage unit **1715**. The machine executable or machine readable code can be provided in the form of software. During use, the code can be executed by the processor **1705**. In some cases, the code can be retrieved from the storage unit **1715** and stored on the memory **1710** for ready access by the processor **1705**. In some situations, the electronic storage unit **1715** can be precluded, and machine-executable instructions are stored on memory **1710**.

The code can be pre-compiled and configured for use with a machine having a processor adapted to execute the code, or can be compiled during runtime. The code can be supplied in a programming language that can be selected to enable the code to execute in a pre-compiled or as-compiled fashion.

Aspects of the systems and methods provided herein, such as the computer system **1701**, can be embodied in programming. Various aspects of the technology may be thought of as "products" or "articles of manufacture" typically in the form of machine (or processor) executable code and/or associated data that is carried on or embodied in a type of machine readable medium. Machine-executable code can be stored on an electronic storage unit, such as memory (e.g., read-only memory, random-access memory, flash memory) or a hard disk. "Storage" type media can include any or all of the tangible memory of the computers, processors or the like, or associated modules thereof, such as various semiconductor memories, tape drives, disk drives and the like, which may provide non-transitory storage at any time for the software programming. All or portions of the software may at times be communicated through the Internet or various other telecommunication networks. Such communications, for example, may enable loading of the software from one computer or processor into another, for example, from a management server or host computer into the computer platform of an application server. Thus, another type of media that may bear the software elements includes optical, electrical and electromagnetic waves, such as used across physical interfaces between local devices, through wired and optical landline networks and over various air-links. The physical elements that carry such waves, such as wired or wireless links, optical links or the like, also may be considered as media bearing the software. As used herein, unless restricted to non-transitory, tangible "storage" media, terms such as computer or machine "readable medium" refer to any medium that participates in providing instructions to a processor for execution.

Hence, a machine readable medium, such as computer-executable code, may take many forms, including but not limited to, a tangible storage medium, a carrier wave medium or physical transmission medium. Non-volatile storage media include, for example, optical or magnetic disks, such as any of the storage devices in any computer(s) or the like, such as may be used to implement the databases, etc. shown in the drawings. Volatile storage media include dynamic memory, such as main memory of such a computer platform. Tangible transmission media include coaxial cables; copper wire and fiber optics, including the wires that comprise a bus within a computer system. Carrier-wave transmission media may take the form of electric or electromagnetic signals, or acoustic or light waves such as those generated during radio frequency (RF) and infrared (IR) data communications. Common forms of computer-readable media therefore include for example: a floppy disk, a flexible disk, hard disk, magnetic tape, any other magnetic medium, a CD-ROM, DVD or DVD-ROM, any other optical medium, punch cards paper tape, any other physical storage medium with patterns of holes, a RAM, a ROM, a PROM and EPROM, a FLASH-EPROM, any other memory chip or cartridge, a carrier wave transporting data or instructions, cables or links transporting such a carrier wave, or any other medium from which a computer may read programming code and/or data. Many of these forms of computer readable media may be involved in carrying one or more sequences of one or more instructions to a processor for execution.

The computer system **1701** can include or be in communication with an electronic display **1735** that comprises a user interface (UI) **1740** for providing, for example, results of nucleic acid sequencing, analysis of nucleic acid sequencing data, characterization of nucleic acid sequencing samples, cell characterizations, etc. Examples of UI's include, without limitation, a graphical user interface (GUI) and web-based user interface.

Methods and systems of the present disclosure can be implemented by way of one or more algorithms. An algorithm can be implemented by way of software upon execution by the central processing unit **1705**. The algorithm can, for example, initiate nucleic acid sequencing, process nucleic acid sequencing data, interpret nucleic acid sequencing results, characterize nucleic acid samples, characterize cells, etc.

VIII. Examples

Example I Cellular RNA Analysis Using Emulsions

In an example, reverse transcription with template switching and cDNA amplification (via PCR) is performed in emulsion droplets with operations as shown in FIG. **9**A. The reaction mixture that is partitioned for reverse transcription and cDNA amplification (via PCR) includes 1,000 cells or 10,000 cells or 10 ng of RNA, beads bearing barcoded oligonucleotides/0.2% Tx-100/5× Kapa buffer, 2× Kapa HS HiFi Ready Mix, 4 μM switch oligo, and Smartscribe. Where cells are present, the mixture is partitioned such that a majority or all of the droplets comprise a single cell and single bead. The cells are lysed while the barcoded oligonucleotides are released from the bead, and the poly-T segment of the barcoded oligonucleotide hybridizes to the poly-A tail of mRNA that is released from the cell as in operation **950**. The poly-T segment is extended in a reverse transcription reaction as in operation **952** and the cDNA transcript is amplified as in operation **954**. The thermal cycling conditions are 42° C. for 130 minutes; 98° C. for 2 min; and 35 cycles of the following 98° C. for 15 sec, 60° C. for 20 sec, and 72° C. for 6 min. Following thermal cycling, the emulsion is broken and the transcripts are purified with Dynabeads and 0.6×SPRI as in operation **956**.

The yield from template switch reverse transcription and PCR in emulsions is shown for 1,000 cells in FIG. **13**A and 10,000 cells in FIG. **13**C and 10 ng of RNA in FIG. **13**B (Smartscribe line). The cDNA transcripts from RT and PCR

performed in emulsions for 10 ng RNA is sheared and ligated to functional sequences, cleaned up with 0.8×SPRI, and is further amplified by PCR as in operation **958**. The amplification product is cleaned up with 0.8×SPRI. The yield from this processing is shown in FIG. **13**B (SSII line).

Example II Cellular RNA Analysis Using Emulsions

In another example, reverse transcription with template switching and cDNA amplification (via PCR) is performed in emulsion droplets with operations as shown in FIG. **9**A. The reaction mixture that is partitioned for reverse transcription and cDNA amplification (via PCR) includes Jurkat cells, beads bearing barcoded oligonucleotides/0.2% TritonX-100/5× Kapa buffer, 2× Kapa HS HiFi Ready Mix, 4 μM switch oligo, and Smartscribe. The mixture is partitioned such that a majority or all of the droplets comprise a single cell and single bead. The cells are lysed while the barcoded oligonucleotides are released from the bead, and the poly-T segment of the barcoded oligonucleotide hybridizes to the poly-A tail of mRNA that is released from the cell as in operation **950**. The poly-T segment is extended in a reverse transcription reaction as in operation **952** and the cDNA transcript is amplified as in operation **954**. The thermal cycling conditions are 42° C. for 130 minutes; 98° C. for 2 min; and 35 cycles of the following 98° C. for 15 sec, 60° C. for 20 sec, and 72° C. for 6 min. Following thermal cycling, the emulsion is broken and the transcripts are cleaned-up with Dynabeads and 0.6×SPRI as in operation **956**. The yield from reactions with various cell numbers (625 cells, 1,250 cells, 2,500 cells, 5,000 cells, and 10,000 cells) is shown in FIG. **14**A. These yields are confirmed with GADPH qPCR assay results shown in FIG. **14**B.

Example III RNA Analysis Using Emulsions

In another example, reverse transcription is performed in emulsion droplets and cDNA amplification is performed in bulk in a manner similar to that as shown in FIG. **9**C. The reaction mixture that is partitioned for reverse transcription includes beads bearing barcoded oligonucleotides, 10 ng Jurkat RNA (e.g., Jurkat mRNA), 5× First-Strand buffer, and Smartscribe. The barcoded oligonucleotides are released from the bead, and the poly-T segment of the barcoded oligonucleotide hybridizes to the poly-A tail of the RNA as in operation **961**. The poly-T segment is extended in a reverse transcription reaction as in operation **963**. The thermal cycling conditions for reverse transcription are one cycle at 42° C. for 2 hours and one cycle at 70° C. for 10 min. Following thermal cycling, the emulsion is broken and RNA and cDNA transcripts are denatured as in operation **962**. A second strand is then synthesized by primer extension with a primer having a biotin tag as in operation **964**. The reaction conditions for this primer extension include cDNA transcript as the first strand and biotinylated extension primer ranging in concentration from 0.5-3.0 μM. The thermal cycling conditions are one cycle at 98° C. for 3 min and one cycle of 98° C. for 15 sec, 60° C. for 20 sec, and 72° C. for 30 min. Following primer extension, the second strand is pulled down with Dynabeads MyOne Streptavidin C1 and T1, and cleaned-up with Agilent SureSelect XT buffers. The second strand is pre-amplified via PCR as in operation **965** with the following cycling conditions—one cycle at 98° C. for 3 min and one cycle of 98° C. for 15 sec, 60° C. for 20 sec, and 72° C. for 30 min. The yield for various concentrations of biotinylated primer (0.5 μM, 1.0 μM, 2.0 μM, and 3.0 μM) is shown in FIG. **15**.

Example IV RNA Analysis Using Emulsions

In another example, in vitro transcription by T7 polymerase is used to produce RNA transcripts as shown in FIG. **10**. The mixture that is partitioned for reverse transcription includes beads bearing barcoded oligonucleotides which also include a T7 RNA polymerase promoter sequence, 10 ng human RNA (e.g., human mRNA), 5× First-Strand buffer, and Smartscribe. The mixture is partitioned such that a majority or all of the droplets comprise a single bead. The barcoded oligonucleotides are released from the bead, and the poly-T segment of the barcoded oligonucleotide hybridizes to the poly-A tail of the RNA as in operation **1050**. The poly-T segment is extended in a reverse transcription reaction as in operation **1052**. The thermal cycling conditions are one cycle at 42° C. for 2 hours and one cycle at 70° C. for 10 min. Following thermal cycling, the emulsion is broken and the remaining operations are performed in bulk. A second strand is then synthesized by primer extension as in operation **1054**. The reaction conditions for this primer extension include cDNA transcript as template and extension primer. The thermal cycling conditions are one cycle at 98° C. for 3 min and one cycle of 98° C. for 15 sec, 60° C. for 20 sec, and 72° C. for 30 min. Following this primer extension, the second strand is purified with 0.6×SPRI. As in operation **1056**, in vitro transcription is then performed to produce RNA transcripts. In vitro transcription is performed overnight, and the transcripts are purified with 0.6×SPRI. The RNA yields from in vitro transcription are shown in FIG. **16**.

While some embodiments of the present invention have been shown and described herein, it will be obvious to those skilled in the art that such embodiments are provided by way of example only. It is not intended that the invention be limited by the specific examples provided within the specification. While the invention has been described with reference to the aforementioned specification, the descriptions and illustrations of the embodiments herein are not meant to be construed in a limiting sense. Numerous variations, changes, and substitutions will now occur to those skilled in the art without departing from the invention. Furthermore, it shall be understood that all aspects of the invention are not limited to the specific depictions, configurations or relative proportions set forth herein which depend upon a variety of conditions and variables. It should be understood that various alternatives to the embodiments of the invention described herein may be employed in practicing the invention. It is therefore contemplated that the invention shall also cover any such alternatives, modifications, variations or equivalents. It is intended that the following claims define the scope of the invention and that methods and structures within the scope of these claims and their equivalents be covered thereby.

What is claimed is:

1. A method for processing messenger ribonucleic acid (mRNA) molecules from a single cell, comprising:

(a) partitioning a plurality of cells and a plurality of beads in a microwell array comprising a plurality of wells, wherein a well of said plurality of wells comprises said single cell from said plurality of cells and a single bead from said plurality of beads, and wherein said single bead comprises nucleic acid barcode molecules each comprising a common barcode sequence;

(b) in said well comprising said single cell and said single bead, releasing messenger ribonucleic acid (mRNA) molecules from said single cell, wherein upon release from said single cell, said released mRNA molecules attach to said nucleic acid barcode molecules;

(c) subjecting said released mRNA molecules attached to said nucleic acid barcode molecules to reverse transcription to yield complementary deoxyribonucleic acid (cDNA) molecules each comprising said common barcode sequence or a complement thereof; and

(d) subjecting said cDNA molecules to one or more reactions to generate a set of nucleic acid molecules for nucleic acid sequencing.

2. The method of claim **1**, wherein (c) is performed in said well comprising said single cell and said single bead, and wherein subsequent to (c), said cDNA molecules, or derivatives thereof, are removed from said well.

3. The method of claim **1**, wherein, prior to (c), said released mRNA molecules attached to said nucleic acid barcode molecules are removed from said well comprising said single cell and said single bead.

4. The method of claim **1**, wherein each of said nucleic acid barcode molecules comprises a universal primer sequence.

5. The method of claim **1**, wherein said single bead is a single magnetic bead.

6. The method of claim **5**, wherein said nucleic acid barcode molecules are attached to said single magnetic bead.

7. The method of claim **6**, wherein prior to (c), said single magnetic bead comprising said released mRNA molecules attached to said nucleic acid barcode molecules are removed from said well comprising said single cell and said single bead.

8. The method of claim **7**, wherein said single magnetic bead is removed using a magnetic field.

9. The method of claim **1**, wherein each of said nucleic acid barcode molecules comprises a sequence for priming the synthesis of cDNA.

10. The method of claim **1**, wherein said plurality of cells comprises at least 100 cells.

11. The method of claim **1**, wherein said plurality of cells comprises at least 1,000 cells.

12. The method of claim **1**, wherein said plurality of cells comprises at least 10,000 cells.

13. The method of claim **1**, wherein said released mRNA molecules attach to said nucleic acid barcode molecules by hybridization.

14. The method of claim **1**, wherein said plurality of beads comprises a plurality of nucleic acid barcode molecules comprising barcode sequences that are different across said plurality of beads.

15. The method of claim **1**, further comprising, prior to (c), (i) pooling said released mRNA molecules attached to said nucleic acid barcode molecules and (ii) performing said one or more reactions in bulk.

16. The method of claim **1**, wherein said one or more reactions comprise nucleic acid amplification that generates amplified products from said plurality of cDNA molecules.

17. The method of claim **16**, wherein said nucleic acid amplification adds functional sequences to said amplified products, wherein said functional sequences permit attachment of said amplified products to a flow cell of a sequencer for said nucleic acid sequencing.

18. The method of claim **16**, further comprising ligating functional sequences to said amplified products, wherein said functional sequences permit attachment of said amplified products to a flow cell of a sequencer for said nucleic acid sequencing.

19. The method of claim **16**, wherein said nucleic acid amplification is polymerase chain reaction.

20. The method of claim **1**, wherein said one or more reactions comprise addition of functional sequences to said plurality of cDNA molecules, wherein said functional sequences permit attachment to a flow cell of a sequencer for said nucleic acid sequencing.

21. The method of claim **1**, further comprising performing said nucleic acid sequencing on said set of nucleic acid molecules, or derivatives thereof, to generate a plurality of sequences comprising sequences corresponding to said released mRNA molecules and said common barcode sequence.

22. The method of claim **1**, wherein said plurality of beads have substantially monodisperse cross-sectional dimensions.

23. The method of claim **1**, wherein said nucleic acid barcode molecules further comprise functional sequences that facilitate sequencing of said set of nucleic acid molecules.

24. The method of claim **4**, wherein said universal primer sequence is a random N-mer.

25. The method of claim **1**, wherein said single bead is a single gel bead.

26. The method of claim **1**, wherein said microwell array comprises 100,000 wells.

27. The method of claim **1**, wherein said microwell array comprises 200,000 wells.

28. The method of claim **1**, wherein said microwell array comprises 1,000,000 wells.

29. The method of claim **1**, wherein a subset of said plurality of wells in said microwell array does not include a cell.

30. The method of claim **1**, wherein a subset of said plurality of wells in said microwell array does not include a bead.

31. The method of claim **25**, wherein said nucleic acid barcode molecules are attached to said single gel bead.

32. The method of claim **25**, wherein prior to (c), said single gel bead comprising said released mRNA molecules attached to said nucleic acid barcode molecules is removed from said well.

33. The method of claim **1**, wherein releasing said mRNA molecules from said single cell comprises lysing said single cell.

34. The method of claim **33**, wherein lysing said single cell comprises treating said single cell with a detergent.

35. The method of claim **33**, wherein lysing said single cell comprises heating said single cell.

36. The method of claim **1**, wherein said nucleic acid barcode molecules comprise a sequence complementary to said released mRNA molecules.

37. The method of claim **1**, wherein said plurality of cells is a plurality of cancer cells.

38. The method of claim **1**, wherein said nucleic acid barcode molecules comprise uracil.

39. The method of claim **1**, wherein said nucleic acid barcode molecules comprise 1,000 nucleic acid barcode molecules.

40. The method of claim **1**, wherein said nucleic acid barcode molecules comprise 100,000 nucleic acid barcode molecules.

41. The method of claim **1**, wherein said plurality of beads comprises 1,000 beads.

42. The method of claim **1**, wherein said plurality of beads comprises 100,000 beads.

43. The method of claim **1**, wherein said plurality of wells comprises 1,000 wells.

44. The method of claim **1**, wherein said plurality of wells comprises 100,000 wells.

45. The method of claim **1**, wherein said plurality of wells comprises 1,000,000 wells.

46. The method of claim **1**, wherein said well has a volume of less than about 5 nanoliters (nL).

47. The method of claim **1**, wherein said well has a volume of less than about 500 nanoliters (nL).

48. The method of claim **14**, wherein said barcode sequences are capable of distinguishing nucleic acid molecules in different wells of said plurality of wells.

49. A method for processing messenger ribonucleic acid (mRNA) molecules from single cells, comprising:

(a) partitioning a plurality of cells and a plurality of beads in a microwell array comprising a plurality of wells, wherein said plurality of wells comprises 1,000 occupied wells, each occupied well of said 1,000 occupied wells comprising a single cell of said plurality of cells and a single bead of said plurality of beads, and wherein said single bead comprises nucleic acid barcode molecules comprising (i) a common barcode sequence capable of identifying said single cell and (ii) a capture sequence, wherein said common barcode sequence is different for different occupied wells of said 1,000 occupied wells;

(b) releasing messenger ribonucleic acid (mRNA) molecules from single cells of said 1,000 occupied wells, wherein released mRNA molecules in each occupied well of said 1,000 occupied wells are captured by said nucleic acid barcode molecules via said capture sequence subsequent to release;

(c) subjecting said released mRNA molecules in each occupied well of said 1,000 occupied wells to reverse transcription to yield complementary deoxyribonucleic acid (cDNA) molecules comprising said common barcode sequence or complement thereof; and

(d) subjecting cDNA molecules or derivatives thereof generated in each occupied well of said 1,000 occupied wells to one or more nucleic acid amplification reactions to generate a set of barcoded nucleic acid molecules for nucleic acid sequencing.

50. The method of claim **49**, wherein (c) is performed in said plurality of wells, and wherein said cDNA molecules or derivatives thereof are removed from said plurality wells and pooled prior to (d).

51. The method of claim **49**, wherein (c) is performed after removing said released mRNA molecules captured by said nucleic acid barcode molecules from said plurality of wells.

52. The method of claim **51**, further comprising pooling said released mRNA molecules prior to (d).

53. The method of claim **49**, wherein said plurality of beads is a plurality of magnetic beads.

54. The method of claim **53**, wherein magnetic beads of said plurality of magnetic beads are removed from said plurality of wells using a magnetic field.

55. The method of claim **49**, wherein said released mRNA molecules are captured by hybridization of the released mRNA molecule of to the capture sequence.

56. The method of claim **49**, wherein said plurality of wells comprises 5,000 occupied wells.

57. The method of claim **49**, wherein said plurality of wells comprises 10,000 occupied wells.

58. The method of claim **49**, wherein no more than 5% of occupied wells of said 1,000 occupied wells have more than one cell.

59. The method of claim **58**, wherein no more than 1% of occupied wells of said 1,000 occupied wells have more than one cell.

60. The method of claim **49**, wherein no more than 5% of occupied wells of said 1,000 occupied wells have more than one bead.

61. The method of claim **60**, wherein no more than 1% of occupied wells of said 1,000 occupied wells have more than one bead.

62. The method of claim **49**, wherein said nucleic acid barcode molecules further comprise (iii) an additional barcode sequence configured to aid in quantitation of mRNA molecules during said nucleic acid sequencing.

63. The method of claim **62**, wherein said additional barcode sequence is a random sequence.

64. The method of claim **62**, wherein a bead of said plurality of bead comprises a first nucleic acid barcode molecule having a first additional barcode sequence and a second nucleic acid barcode molecule having a second additional barcode sequence different from said first additional barcode sequence.

65. The method of claim **49**, wherein said one or more nucleic acid amplification reactions add functional sequences to barcoded nucleic acid molecules of said set of nucleic acid molecules that permit binding of said barcoded nucleic acid molecules to a flow cell of a sequencer.

66. The method of claim **49**, further comprising ligating functional sequences to barcoded nucleic acid molecules of said set of barcoded nucleic acid molecules that permit binding of said barcoded nucleic acid molecules to a flow cell of a sequencer.

67. The method of claim **49**, wherein said one or more nucleic acid amplification reactions are polymerase chain reactions.

68. The method of claim **49**, wherein wells of said plurality of wells have a volume of less than about 5 nanoliters (nL).

69. The method of claim **49**, wherein wells of said plurality of wells have a volume of less than about 500 nanoliters (nL).

70. The method of claim **49**, wherein said microwell array comprises 100,000 wells.

71. The method of claim **49**, wherein said microwell array comprises 200,000 wells.

72. The method of claim **49**, wherein said microwell array comprises 1,000,000 wells.

73. The method of claim **49**, wherein a subset of said plurality of wells in said microwell array does not include a cell.

74. The method of claim **49**, wherein a subset of said plurality of wells in said microwell array does not include a bead.

* * * * *

Exhibit D

US010280459B1

(12) **United States Patent**
Brenner et al.

(10) **Patent No.: US 10,280,459 B1**
(45) **Date of Patent: *May 7, 2019**

(54) **METHODS FOR ANALYZING NUCLEIC ACIDS FROM SINGLE CELLS**

(71) Applicant: **10X Genomics, Inc.**, Pleasanton, CA (US)

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(73) Assignee: **10X GENOMICS, INC.**, Pleasanton, CA (US)

(*) Notice: Subject to any disclaimer, the term of this patent is extended or adjusted under 35 U.S.C. 154(b) by 0 days.

This patent is subject to a terminal disclaimer.

(21) Appl. No.: **16/261,268**

(22) Filed: **Jan. 29, 2019**

Related U.S. Application Data

(63) Continuation of application No. 16/194,047, filed on Nov. 16, 2018, which is a continuation of application No. 15/677,957, filed on Aug. 15, 2017, now Pat. No. 10,155,981, which is a continuation of application No. 14/792,094, filed on Jul. 6, 2015, now abandoned, which is a continuation of application No. 14/172,694, filed on Feb. 4, 2014, now Pat. No. 9,102,980, which is a continuation of application No. 14/021,790, filed on Sep. 9, 2013, now Pat. No. 8,679,756, which is a continuation of application No. 13/859,450, filed on Apr. 9, 2013, now Pat. No. 8,563,274, which is a continuation of application No. 13/622,872, filed on Sep. 19, 2012, now abandoned, which is a continuation of application No. 13/387,343, filed as application No. PCT/IB2010/002243 on Aug. 13, 2010, now Pat. No. 8,298,767.

(60) Provisional application No. 61/288,792, filed on Dec. 21, 2009, provisional application No. 61/235,595, filed on Aug. 20, 2009.

(51) **Int. Cl.**

C12Q 1/6874	(2018.01)
C12N 15/10	(2006.01)
C12Q 1/6806	(2018.01)
C12Q 1/6855	(2018.01)
C12Q 1/686	(2018.01)

(52) **U.S. Cl.**
CPC ***C12Q 1/6874*** (2013.01); ***C12N 15/1065*** (2013.01); ***C12Q 1/6806*** (2013.01); ***C12Q 1/686*** (2013.01); ***C12Q 1/6855*** (2013.01)

(58) **Field of Classification Search**
CPC C12Q 1/6874; C12N 15/1065
USPC 506/16
See application file for complete search history.

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(Continued)

Primary Examiner — Karla A Dines
(74) *Attorney, Agent, or Firm* — Morgan, Lewis & Bockius LLP

(57) **ABSTRACT**

Aspects of the present invention include analyzing nucleic acids from single cells using methods that include using tagged polynucleotides containing multiplex identifier sequences.

30 Claims, 13 Drawing Sheets

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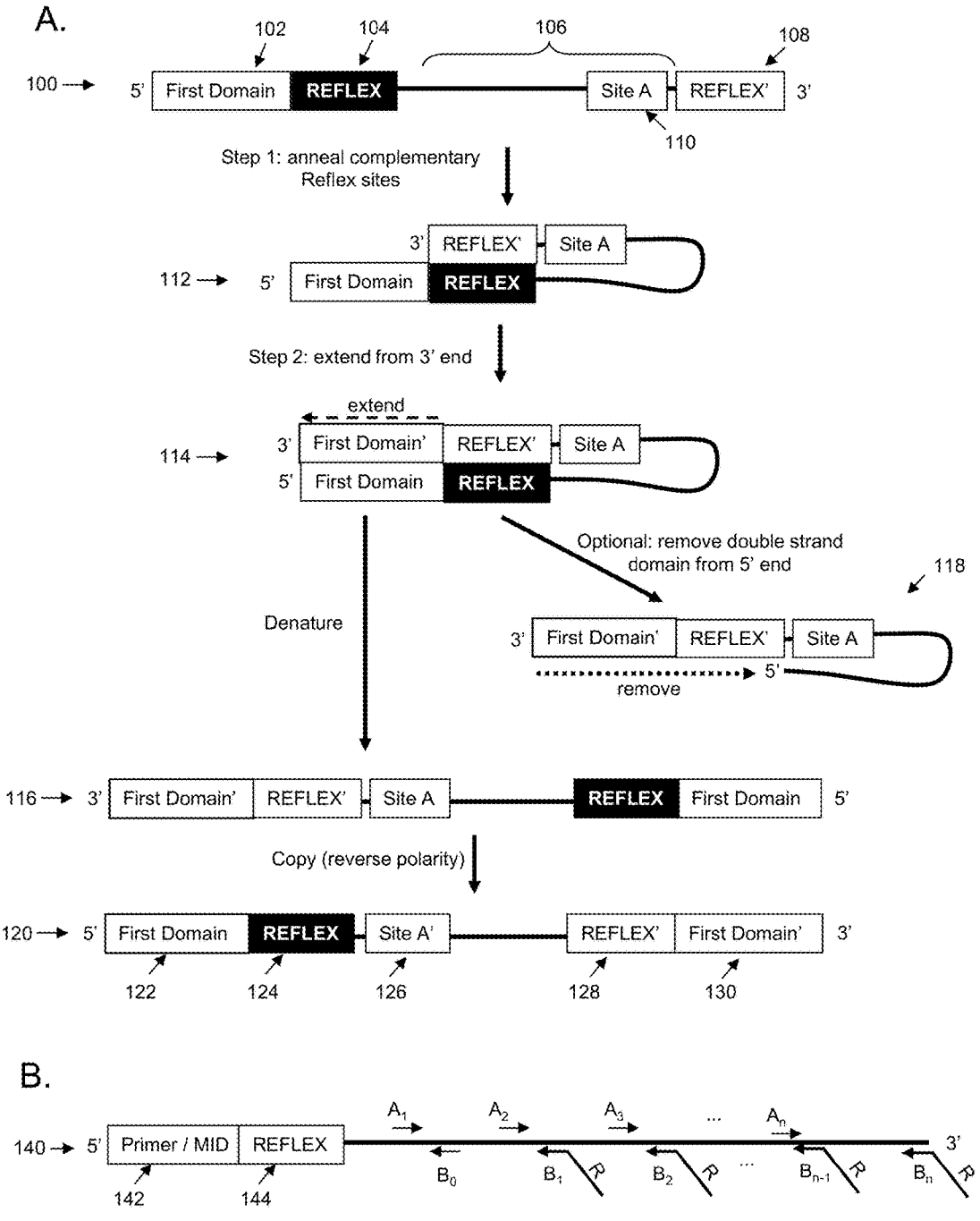

A.
102
104
106
108
100
5'
First Domain
REFLEX
Site A
REFLEX'
3'
110
Step 1: anneal complementary Reflex sites
3'
REFLEX'
Site A
112
5'
First Domain
REFLEX
Step 2: extend from 3' end
extend
114
3'
First Domain'
REFLEX'
Site A
5'
First Domain
REFLEX
Optional: remove double strand domain from 5' end
118
Denature
3'
First Domain'
REFLEX'
Site A
5'
remove
116
3'
First Domain'
REFLEX'
Site A
REFLEX
First Domain
5'
Copy (reverse polarity)
120
5'
First Domain
REFLEX
Site A'
REFLEX'
First Domain'
3'
122
124
126
128
130
B.
140
5'
Primer / MID
REFLEX
142
144
A1
A2
A3
An
B0
B1
B2
Bn-1
Bn
P
3'

Fig. 1

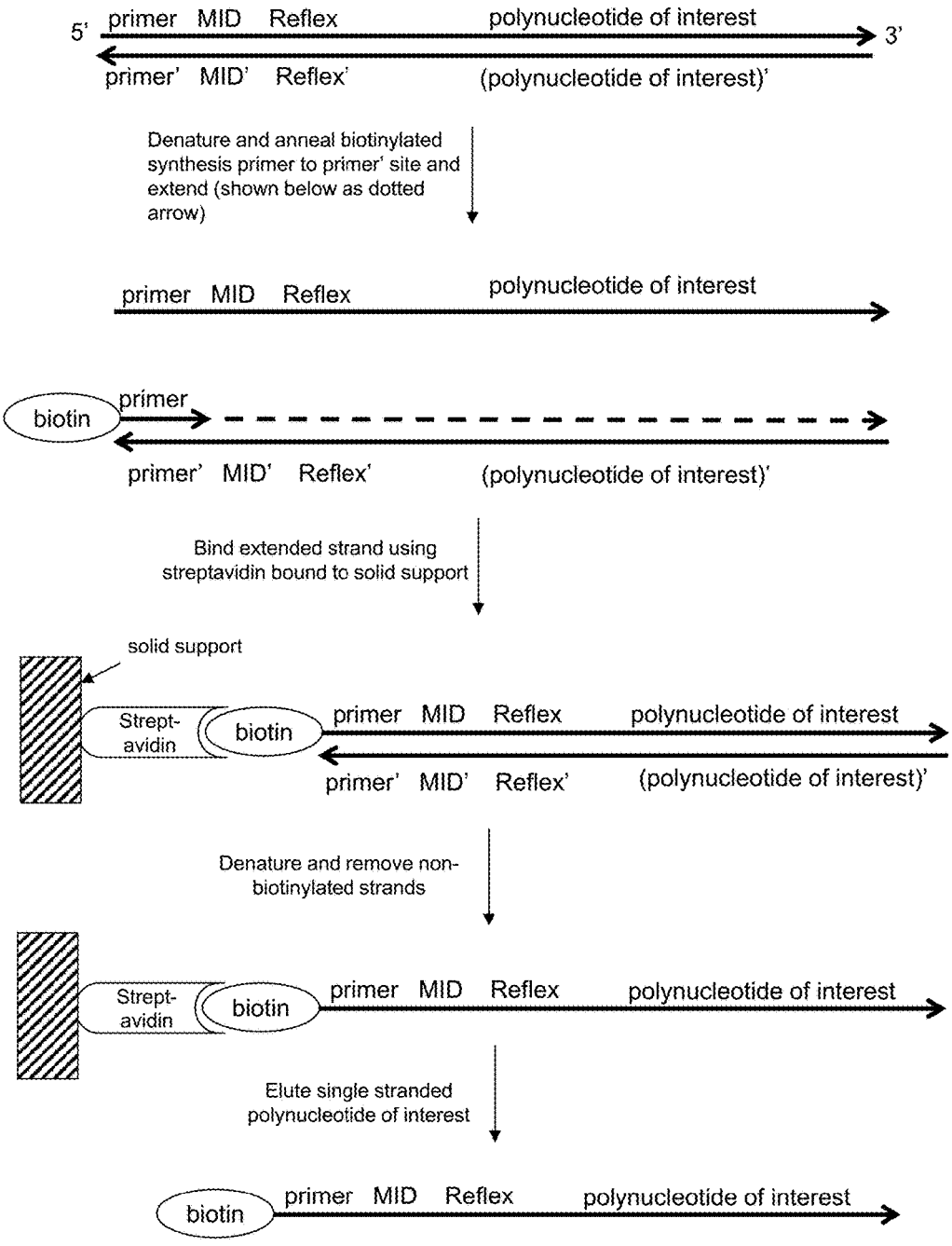
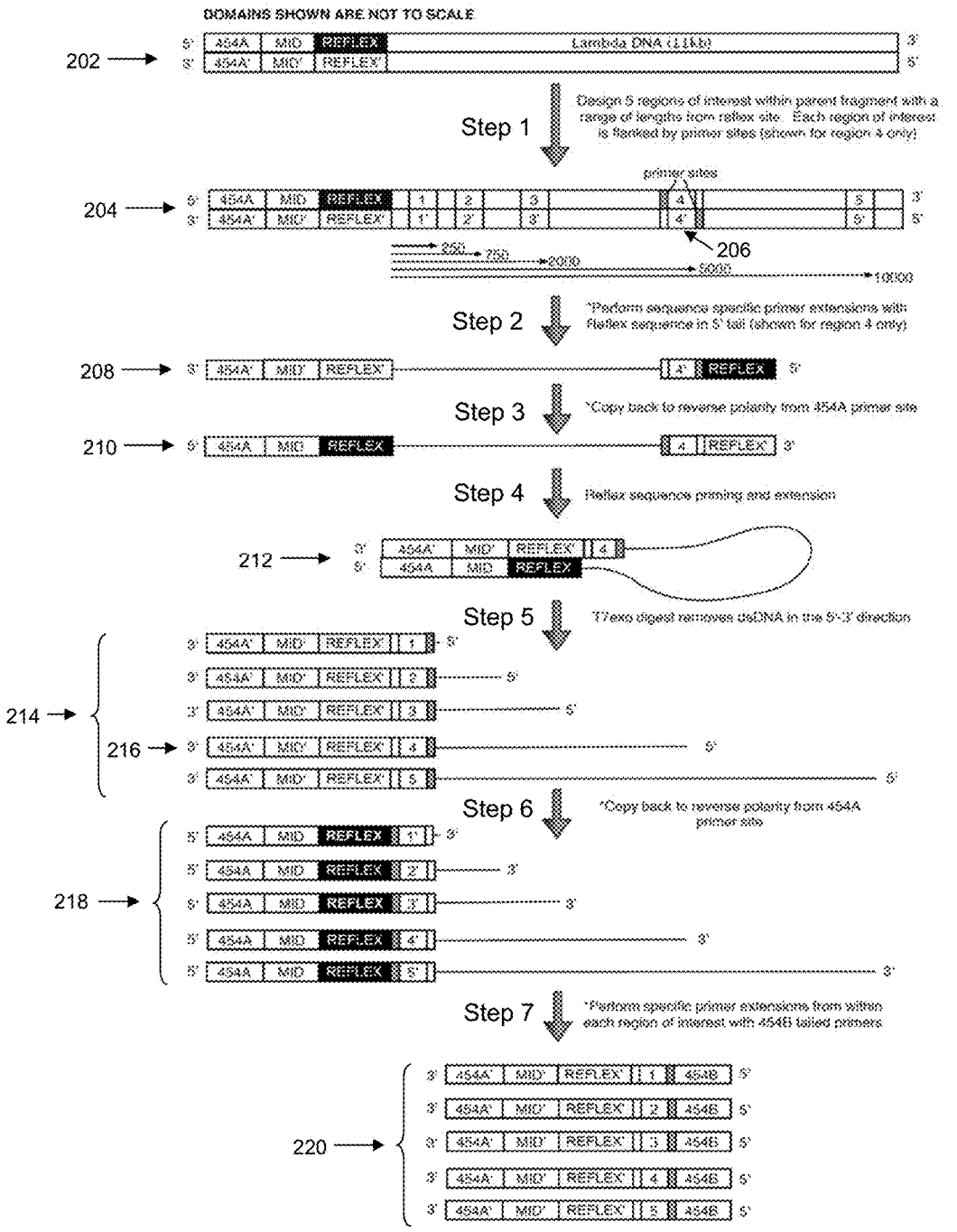

5'
primer MID Reflex
polynucleotide of interest
3'
primer' MID' Reflex'
(polynucleotide of interest)'
Denature and anneal biotinylated synthesis primer to primer' site and extend (shown below as dotted arrow)
primer MID Reflex
polynucleotide of interest
biotin
primer
primer' MID' Reflex'
(polynucleotide of interest)'
Bind extended strand using streptavidin bound to solid support
solid support
Strept-avidin
biotin
primer MID Reflex
polynucleotide of interest
primer' MID' Reflex'
(polynucleotide of interest)'
Denature and remove non-biotinylated strands
Strept-avidin
biotin
primer MID Reflex
polynucleotide of interest
Elute single stranded polynucleotide of interest
biotin
primer MID Reflex
polynucleotide of interest

Fig. 2



Primer extension reactions with * may be performed such that isolation of single strand species is facilitated (e.g., using primers with binding moieties and/or multiple cycles of extension)

Fig. 3

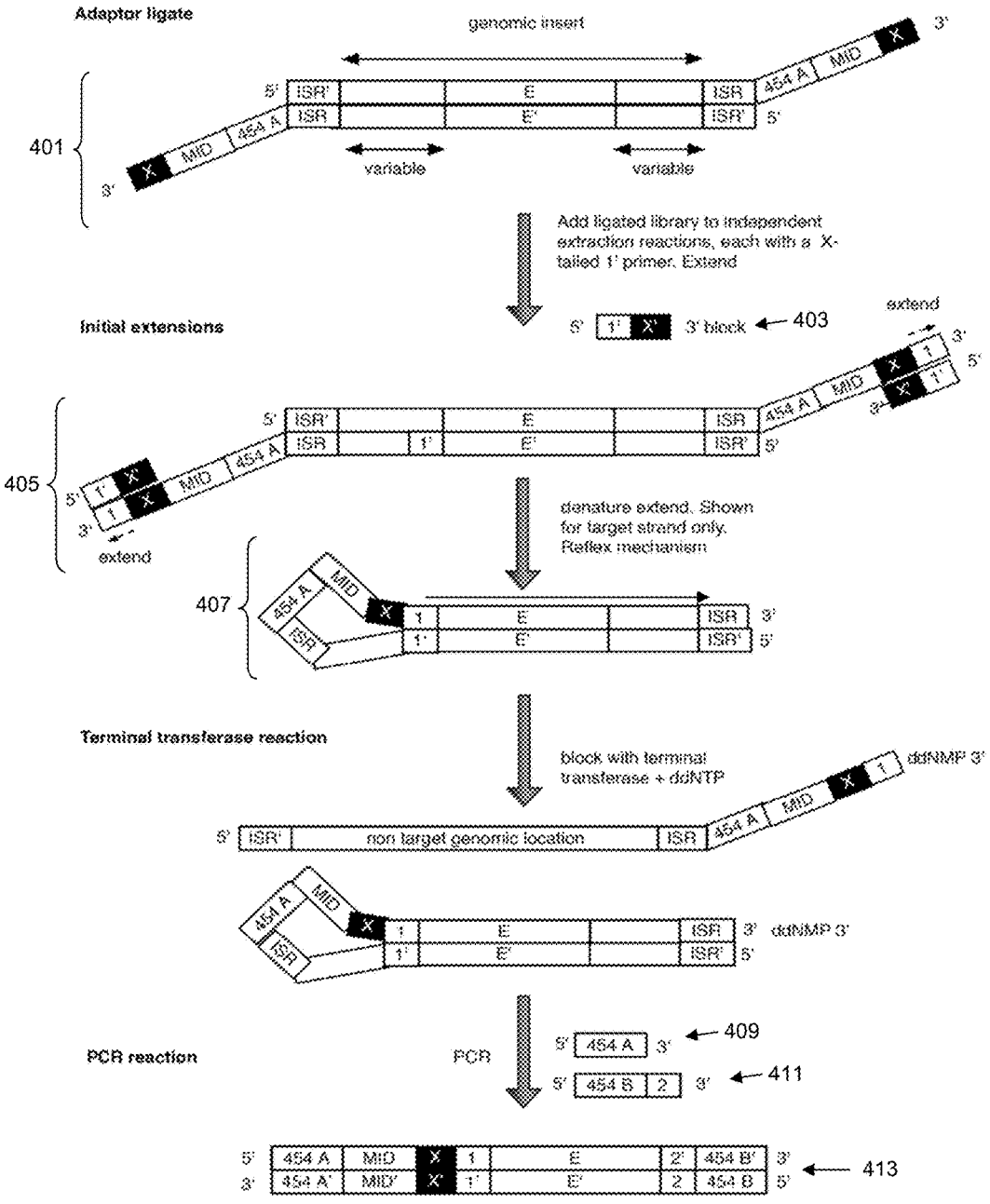

Adaptor ligate
genomic insert
401
variable
variable
Add ligated library to independent extraction reactions, each with a X-tailed 1' primer. Extend
Initial extensions
403
extend
405
extend
denature extend. Shown for target strand only. Reflex mechanism
407
Terminal transferase reaction
block with terminal transferase + ddNTP
ddNMP 3'
non target genomic location
PCR reaction
PCR
409
411
413

Fig. 4

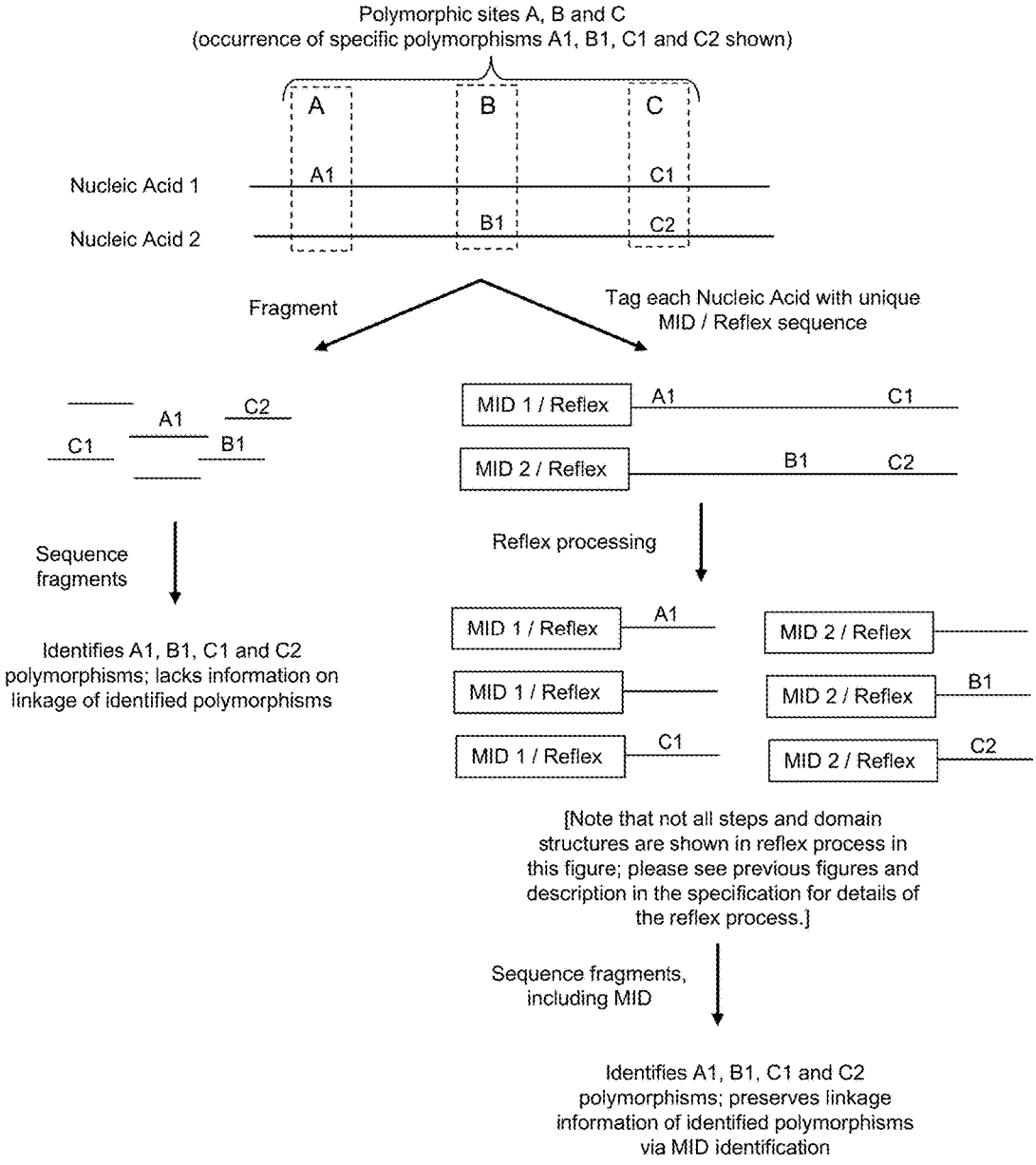
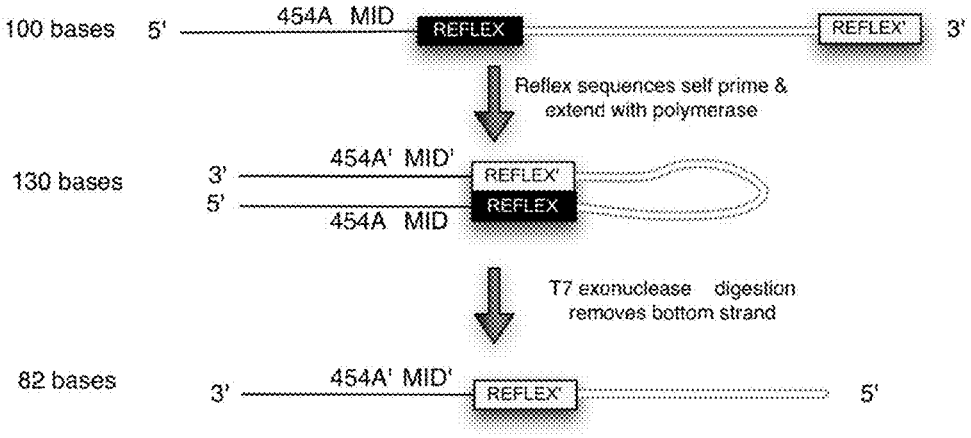
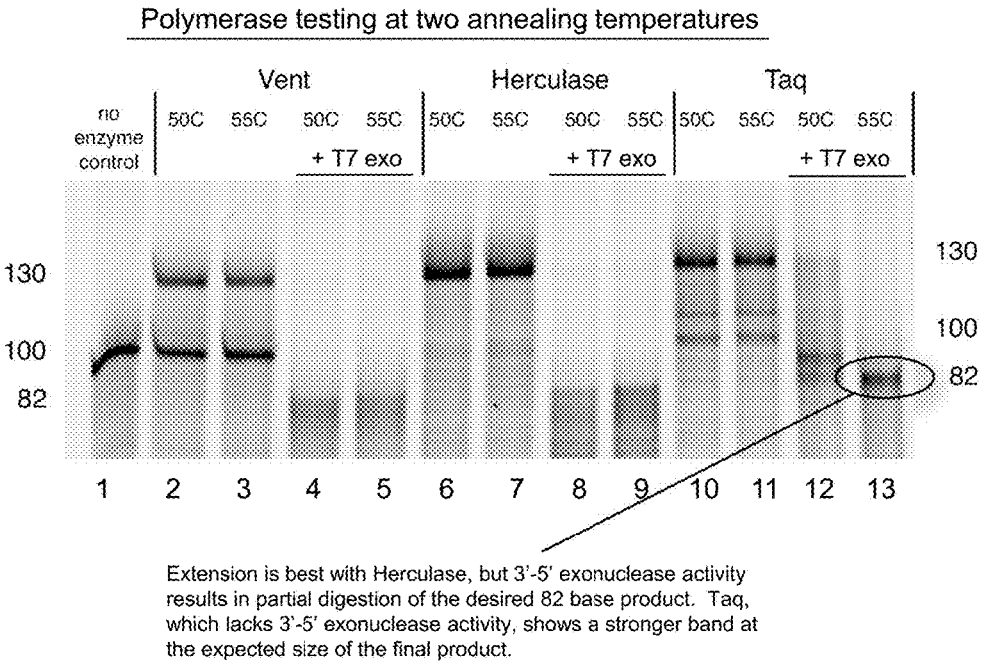

Polymorphic sites A, B and C
(occurrence of specific polymorphisms A1, B1, C1 and C2 shown)
A
B
C
Nucleic Acid 1
A1
C1
Nucleic Acid 2
B1
C2
Fragment
Tag each Nucleic Acid with unique MID / Reflex sequence
A1
C2
C1
B1
MID 1 / Reflex
A1
C1
MID 2 / Reflex
B1
C2
Sequence fragments
Reflex processing
Identifies A1, B1, C1 and C2 polymorphisms; lacks information on linkage of identified polymorphisms
MID 1 / Reflex
A1
MID 2 / Reflex
MID 1 / Reflex
MID 2 / Reflex
B1
MID 1 / Reflex
C1
MID 2 / Reflex
C2
[Note that not all steps and domain structures are shown in reflex process in this figure; please see previous figures and description in the specification for details of the reflex process.]
Sequence fragments, including MID
Identifies A1, B1, C1 and C2 polymorphisms; preserves linkage information of identified polymorphisms via MID identification

Fig. 5

A.



B.



Extension is best with Herculase, but 3'-5' exonuclease activity results in partial digestion of the desired 82 base product. Taq, which lacks 3'-5' exonuclease activity, shows a stronger band at the expected size of the final product.

Fig. 6

A.


5'
454A MID
REFLEX
REFLEX'
3'
+ Competitor
5'
REFLEX'
3'

B.

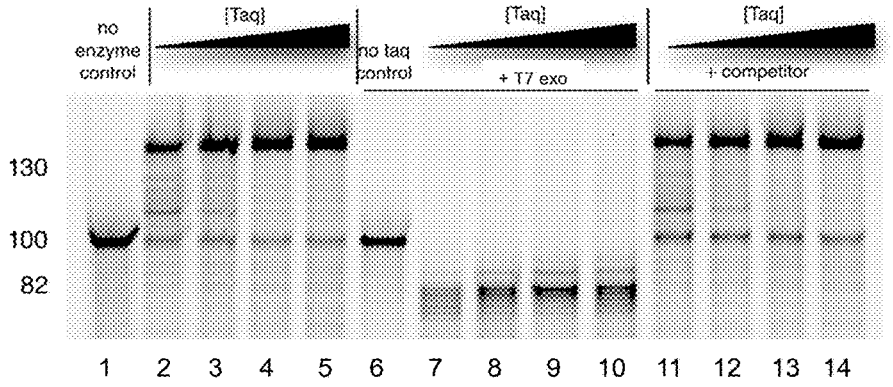
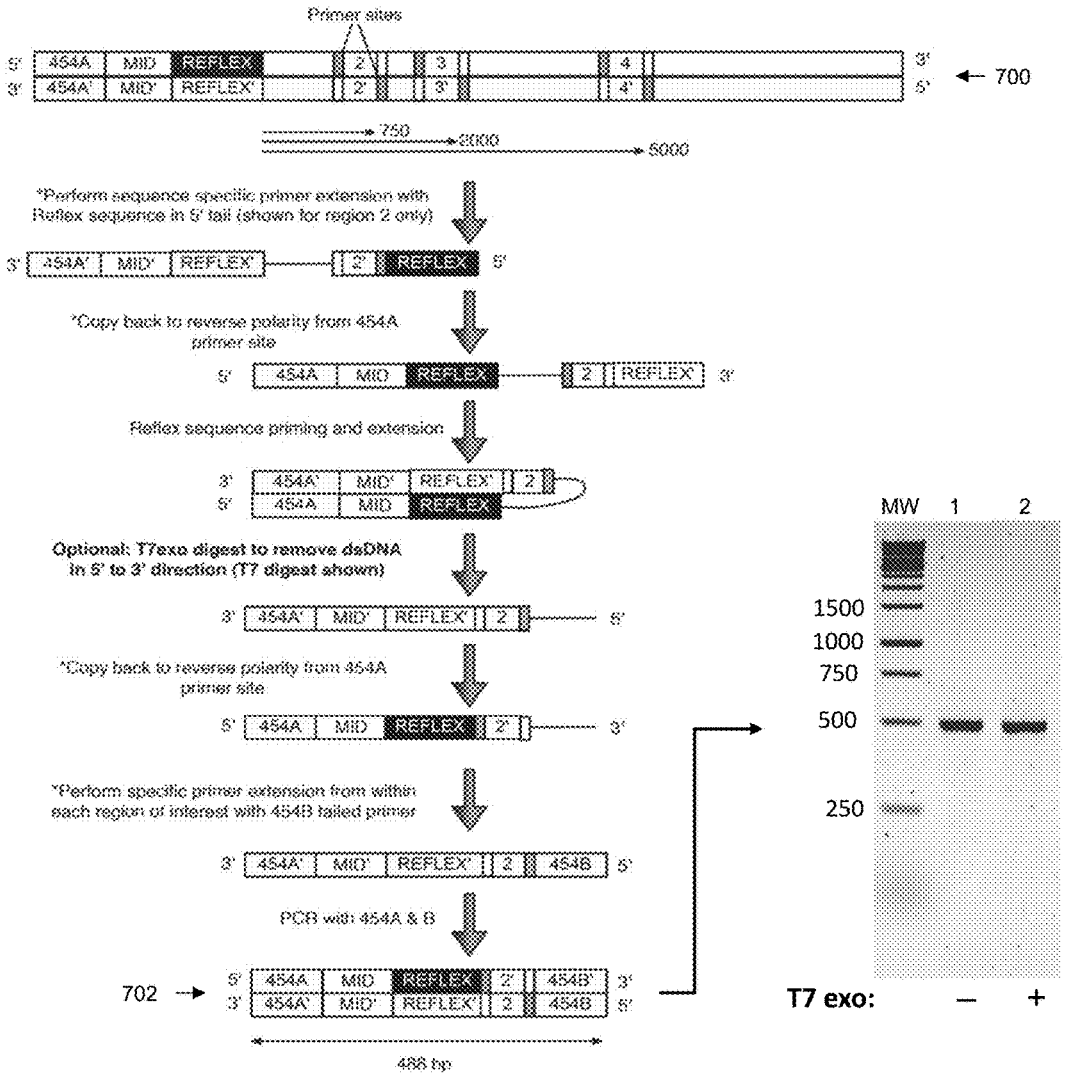

no enzyme control
[Taq]
no taq control
[Taq]
+ T7 exo
[Taq]
+ competitor
130
100
82
1 2 3 4 5 6 7 8 9 10 11 12 13 14

Fig. 7



Primer extension reactions with * may be performed such that isolation of single strand species is facilitated (e.g., using primers with binding moieties and/or multiple cycles of extension)

Fig. 8

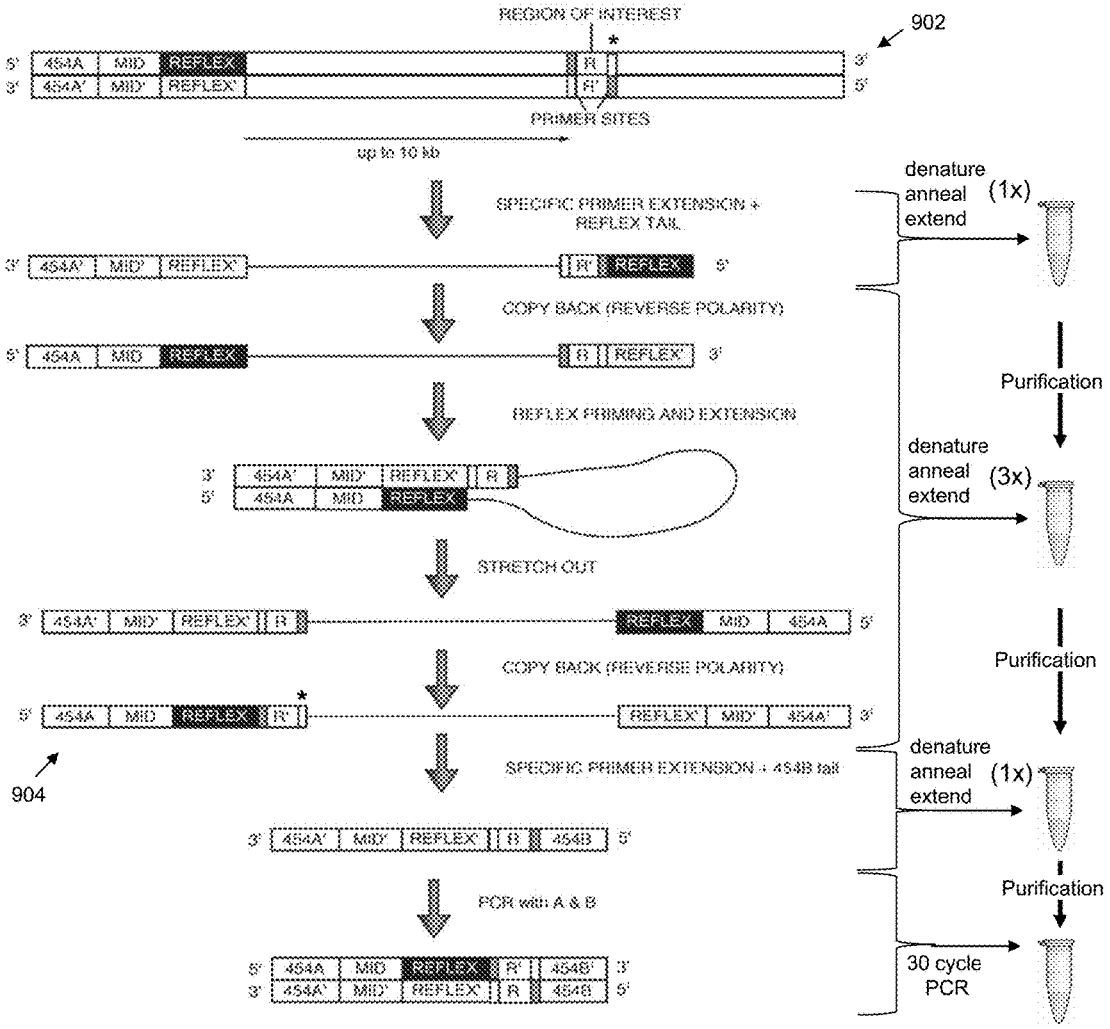

REGION OF INTEREST
902
PRIMER SITES
up to 10 kb
SPECIFIC PRIMER EXTENSION + REFLEX TAIL
COPY BACK (REVERSE POLARITY)
REFLEX PRIMING AND EXTENSION
STRETCH OUT
COPY BACK (REVERSE POLARITY)
SPECIFIC PRIMER EXTENSION + 454B tail
PCR with A & B
904
denature anneal extend (1x)
Purification
denature anneal extend (3x)
Purification
denature anneal extend (1x)
Purification
30 cycle PCR

Fig. 9

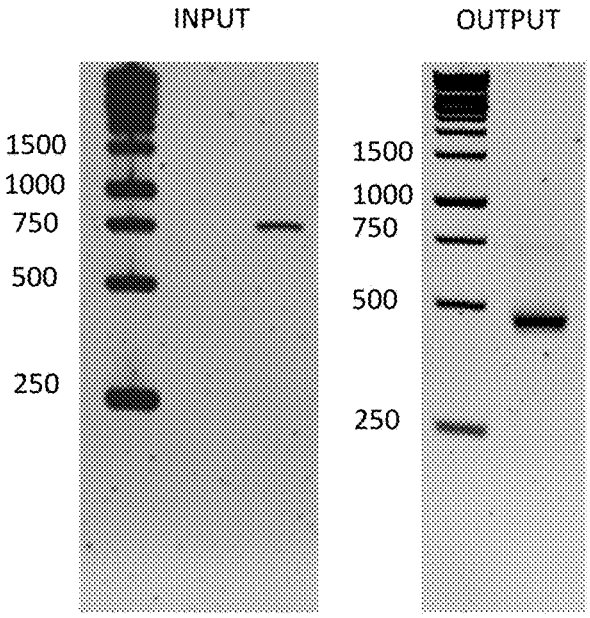
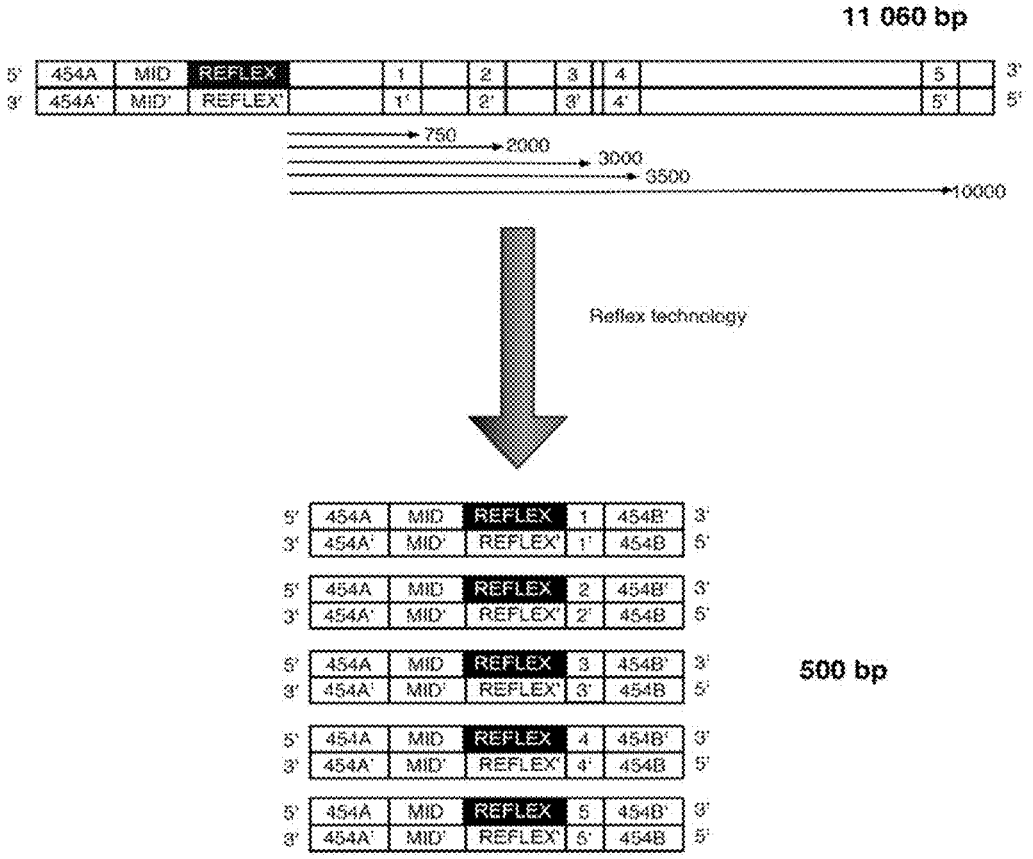


Fig. 10


11 060 bp
5' 454A MID REFLEX 1 2 3 4 5 3'
3' 454A' MID' REFLEX' 1' 2' 3' 4' 5' 5'
750
2000
3000
3500
10000
Reflex technology
5' 454A MID REFLEX 1 454B' 3'
3' 454A' MID' REFLEX' 1' 454B 5'
5' 454A MID REFLEX 2 454B' 3'
3' 454A' MID' REFLEX' 2' 454B 5'
5' 454A MID REFLEX 3 454B' 3'
3' 454A' MID' REFLEX' 3' 454B 5'
500 bp
5' 454A MID REFLEX 4 454B' 3'
3' 454A' MID' REFLEX' 4' 454B 5'
5' 454A MID REFLEX 5 454B' 3'
3' 454A' MID' REFLEX' 5' 454B 5'

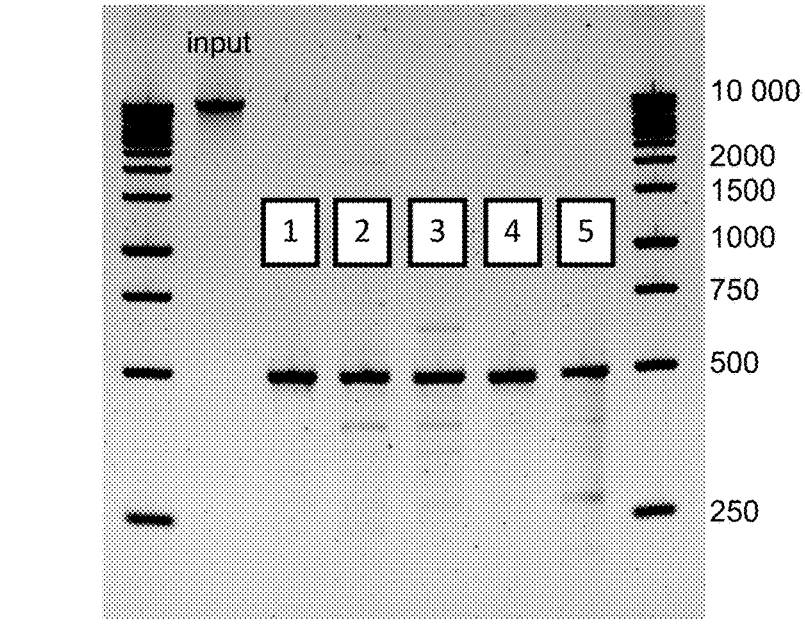
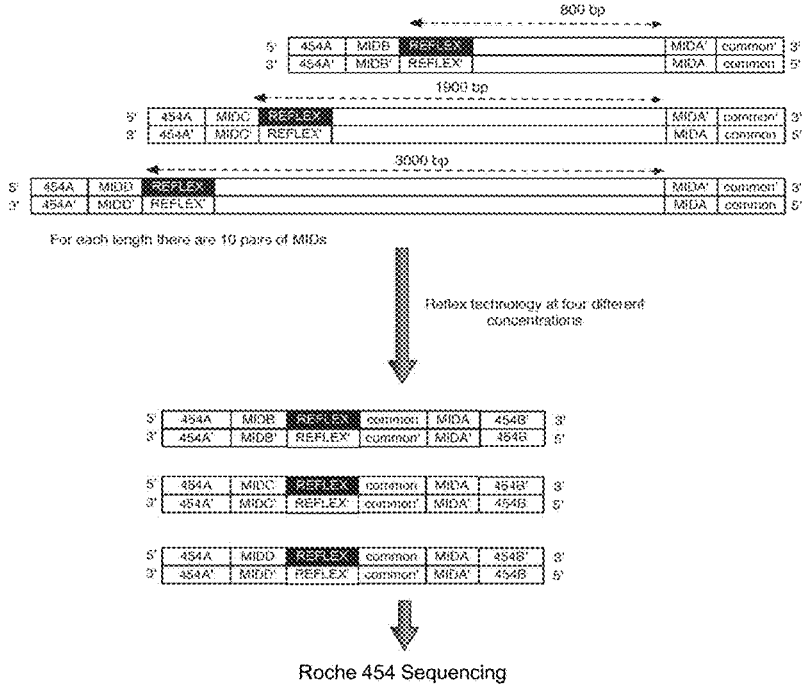

input
1
2
3
4
5
10 000
2000
1500
1000
750
500
250
Lanes: 1 2 3 4 5 6

Fig. 11


800 bp
1900 bp
3000 bp
454A
MIDB
REFLEX
MIDA
common
454B
MIDC
MIDD
For each length there are 10 pairs of MIDs
Reflex technology at four different concentrations
Roche 454 Sequencing

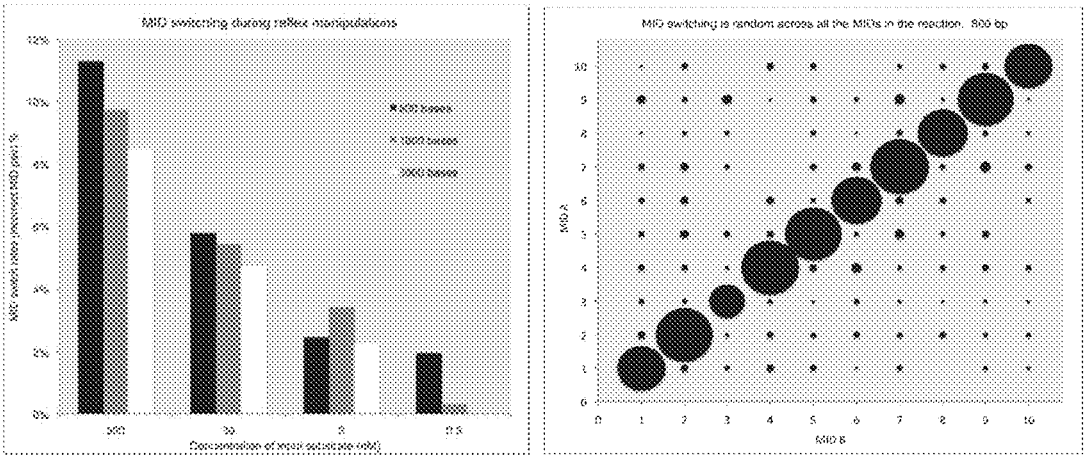

MID switching during reflex manipulations
MID switching is random across all the MIDs in the reaction. 800 bp

Fig. 12

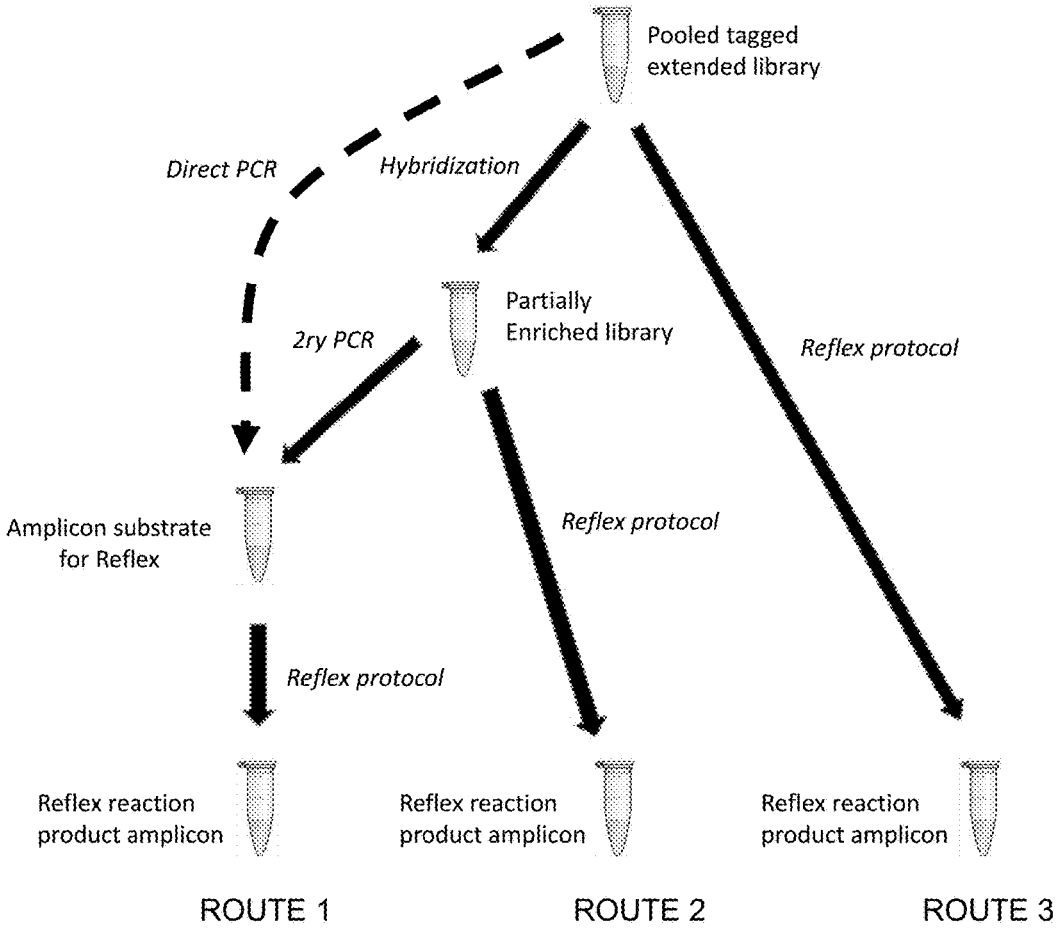

Pooled tagged extended library
Direct PCR
Hybridization
Partially Enriched library
2ry PCR
Reflex protocol
Amplicon substrate for Reflex
Reflex protocol
Reflex protocol
Reflex reaction product amplicon
Reflex reaction product amplicon
Reflex reaction product amplicon
ROUTE 1
ROUTE 2
ROUTE 3

Fig. 13

METHODS FOR ANALYZING NUCLEIC ACIDS FROM SINGLE CELLS

CROSS REFERENCE TO RELATED APPLICATIONS

This application is a continuation of U.S. application Ser. No. 16/194,047, filed Nov. 16, 2018, which is a continuation of U.S. application Ser. No. 15/677,957, filed Aug. 15, 2017, now patent Ser. No. 10/155,981, which is a continuation of U.S. application Ser. No. 14/792,094, filed Jul. 6, 2015, which is a continuation of U.S. application Ser. No. 14/172,694, filed Feb. 4, 2014, now U.S. Pat. No. 9,102,980, which is a continuation of U.S. application Ser. No. 14/021,790, filed Sep. 9, 2013, now U.S. Pat. No. 8,679,756, which is a continuation of U.S. application Ser. No. 13/859,450, filed Apr. 9, 2013, now U.S. Pat. No. 8,563,274, which is a continuation of U.S. application Ser. No. 13/622,872, filed Sep. 19, 2012, which is a continuation of U.S. application Ser. No. 13/387,343, filed Feb. 15, 2012, now U.S. Pat. No. 8,298,767, which is a § 371 National Phase Application of PCT/IB2010/002243, filed Aug. 13, 2010, which claims priority to U.S. Provisional Application No. 61/235,595, filed Aug. 20, 2009 and U.S. Provisional Application No. 61/288,792, filed Dec. 21, 2009; all of which are herein incorporated by reference.

BACKGROUND OF THE INVENTION

We have previously described methods that enable tagging each of a population of fragmented genomes and then combining them together to create a 'population library' that can be processed and eventually sequenced as a mixture. The population tags enable analysis software to parse the sequence reads into files that can be attributed to a particular genome in the population. One limitation of the overall process stems from limitations of existing DNA sequencing technologies. In particular, if fragments in the regions of interest of the genome are longer than the lengths that can be sequenced by a particular technology, then such fragments will not be fully analyzed (since sequencing proceeds from an end of a fragment inward). Furthermore, a disadvantage of any sequencing technology dependent on fragmentation is that sequence changes in one part of a particular genomic region may not be able to be linked to sequence changes in other parts of the same genome (e.g., the same chromosome) because the sequence changes reside on different fragments. (See FIG. **5** and its description below).

The present invention removes the limitations imposed by current sequencing technologies as well as being useful in a number of other nucleic acid analyses.

SUMMARY OF THE INVENTION

Aspects of the present invention are drawn to processes for moving a region of interest in a polynucleotide from a first position to a second position with regard to a domain within the polynucleotide, also referred to as a "reflex method" (or reflex process, reflex sequence process, reflex reaction, and the like). In certain embodiments, the reflex method results in moving a region of interest into functional proximity to specific domain elements present in the polynucleotide (e.g., primer sites and/or MID). Compositions, kits and systems that find use in carrying out the reflex processes described herein are also provided.

BRIEF DESCRIPTION OF THE DRAWINGS

The invention is best understood from the following detailed description when read in conjunction with the accompanying drawings. It is emphasized that, according to common practice, the various features of the drawings are not to scale. Indeed, the dimensions of the various features are arbitrarily expanded or reduced for clarity. Included in the drawings are the following figures:

FIG. **1**: Panel A is a schematic diagram illustrating moving a first domain from one site to another in a nucleic acid molecule using a reflex sequence. Panel B is a schematic diagram depicting the relative position of primer pairs (A_n-B_n primers) that find use in aspects of the reflex process described herein.

FIG. **2** shows an exemplary embodiment of using binding partner pairs (biotin/streptavidin) to isolate single stranded polynucleotides of interest.

FIG. **3** is a schematic diagram illustrating an exemplary embodiment for moving a primer site and a MID to a specific location in a nucleic acid of interest.

FIG. **4** shows a schematic diagram illustrating an exemplary use of the reflex process for generating a sample enriched for fragments having a region of interest (e.g., from a population of randomly fragmented and asymmetrically tagged polynucleotides).

FIG. **5** shows a comparison of methods for identifying nucleic acid polymorphisms in homologous nucleic acids in a sample (e.g., the same region derived from a chromosomal pair of a diploid cell or viral genomes/transcripts). The top schematic shows two nucleic acid molecules in a sample (1 and 2) having a different assortment of polymorphisms in polymorphic sites A, B and C (A1, B1, C1 and C2). Standard sequencing methods using fragmentation (left side) can identify the polymorphisms in these nucleic acids but do not retain linkage information. Employing the reflex process described herein to identify polymorphisms (right side) maintains linkage information.

FIG. **6**: Panel A is a schematic showing expected structures and sizes of nucleic acid species in the reflex process; Panel B is a polyacrylamide gel showing the nucleic acid species produced in the reflex process described in Example 1.

FIG. **7**: Panel A is a schematic showing the structure of the nucleic acid and competitor used in the reflex process; Panel B is a polyacrylamide gel showing the nucleic acid species produced in the reflex process described in Example 1.

FIG. **8** shows a flow chart of a reflex process (left) in which the T7 exonuclease step is optional. The gel on the right shows the resultant product of the reflex process either without the T7 exonuclease step (lane 1) or with the T7 exonuclease step (lane 2).

FIG. **9** shows an exemplary reflex process workflow with indications on the right as to where purification of reaction products is employed (e.g., using Agencourt beads to remove primer oligos).

FIG. **10** shows the starting material (left panel) and the resultant product generated (right panel) using a reflex process without using a T7 exonuclease step (as described in Example II). The reflex site in the starting material is a sequence normally present in the polynucleotide being processed (also called a "non-artificial" reflex site). This figure shows that the 755 base pair starting nucleic acid was processed to the expected 461 base pair product, thus confirming that a "non-artificial" reflex site is effective in transferring an adapter domain from one location to another in a polynucleotide of interest in a sequence specific manner.

FIG. **11** shows a schematic and results of an experiment in which the reflex process is performed on a single large initial template (a "parent" fragment) to generate five different products ("daughter" products) each having a differ-

ent region of interest (i.e., daughter products are produced having either region 1, 2, 3, 4 or 5).

FIG. **12** shows a schematic and results of experiments performed to determine the prevalence of intramolecular rearrangement during the reflex process (as desired) vs. intermolecular rearrangement (MID switching).

FIG. **13** shows a diagram of exemplary workflows for preparing material for and performing the reflex process.

Definitions

Unless otherwise defined, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. Still, certain elements are defined for the sake of clarity and ease of reference.

Terms and symbols of nucleic acid chemistry, biochemistry, genetics, and molecular biology used herein follow those of standard treatises and texts in the field, e.g. Kornberg and Baker, DNA Replication, Second Edition (W.H. Freeman, New York, 1992); Lehninger, Biochemistry, Second Edition (Worth Publishers, New York, 1975); Strachan and Read, Human Molecular Genetics, Second Edition (Wiley-Liss, New York, 1999); Eckstein, editor, Oligonucleotides and Analogs: A Practical Approach (Oxford University Press, New York, 1991); Gait, editor, Oligonucleotide Synthesis: A Practical Approach (IRL Press, Oxford, 1984); and the like.

"Amplicon" means the product of a polynucleotide amplification reaction. That is, it is a population of polynucleotides, usually double stranded, that are replicated from one or more starting sequences. The one or more starting sequences may be one or more copies of the same sequence, or it may be a mixture of different sequences. Amplicons may be produced by a variety of amplification reactions whose products are multiple replicates of one or more target nucleic acids. Generally, amplification reactions producing amplicons are "template-driven" in that base pairing of reactants, either nucleotides or oligonucleotides, have complements in a template polynucleotide that are required for the creation of reaction products. In one aspect, template-driven reactions are primer extensions with a nucleic acid polymerase or oligonucleotide ligations with a nucleic acid ligase. Such reactions include, but are not limited to, polymerase chain reactions (PCRs), linear polymerase reactions, nucleic acid sequence-based amplification (NASBAs), rolling circle amplifications, and the like, disclosed in the following references that are incorporated herein by reference: Mullis et al, U.S. Pat. Nos. 4,683,195; 4,965,188; 4,683,202; 4,800,159 (PCR); Gelfand et al, U.S. Pat. No. 5,210,015 (real-time PCR with "TAQMAN™" probes); Wittwer et al, U.S. Pat. No. 6,174,670; Kacian et al, U.S. Pat. No. 5,399,491 ("NASBA"); Lizardi, U.S. Pat. No. 5,854,033; Aono et al, Japanese patent publ. JP 4-262799 (rolling circle amplification); and the like. In one aspect, amplicons of the invention are produced by PCRs. An amplification reaction may be a "real-time" amplification if a detection chemistry is available that permits a reaction product to be measured as the amplification reaction progresses, e.g. "real-time PCR" described below, or "real-time NASBA" as described in Leone et al, Nucleic Acids Research, 26: 2150-2155 (1998), and like references. As used herein, the term "amplifying" means performing an amplification reaction. A "reaction mixture" means a solution containing all the necessary reactants for performing a reaction, which may include, but not be limited to, buffering agents to maintain pH at a selected level during a reaction, salts, co-factors, scavengers, and the like.

The term "assessing" includes any form of measurement, and includes determining if an element is present or not. The terms "determining", "measuring", "evaluating", "assessing" and "assaying" are used interchangeably and includes quantitative and qualitative determinations. Assessing may be relative or absolute. "Assessing the presence of" includes determining the amount of something present, and/or determining whether it is present or absent. As used herein, the terms "determining," "measuring," and "assessing," and "assaying" are used interchangeably and include both quantitative and qualitative determinations.

Polynucleotides that are "asymmetrically tagged" have left and right adapter domains that are not identical. This process is referred to generically as attaching adapters asymmetrically or asymmetrically tagging a polynucleotide, e.g., a polynucleotide fragment. Production of polynucleotides having asymmetric adapter termini may be achieved in any convenient manner. Exemplary asymmetric adapters are described in: U.S. Pat. Nos. 5,712,126 and 6,372,434; U.S. Patent Publications 2007/0128624 and 2007/0172839; and PCT publication WO/2009/032167; all of which are incorporated by reference herein in their entirety. In certain embodiments, the asymmetric adapters employed are those described in U.S. patent application Ser. No. 12/432,080, filed on Apr. 29, 2009, incorporated herein by reference in its entirety.

As one example, a user of the subject invention may use an asymmetric adapter to tag polynucleotides. An "asymmetric adapter" is one that, when ligated to both ends of a double stranded nucleic acid fragment, will lead to the production of primer extension or amplification products that have non-identical sequences flanking the genomic insert of interest. The ligation is usually followed by subsequent processing steps so as to generate the non-identical terminal adapter sequences. For example, replication of an asymmetric adapter attached fragment(s) results in polynucleotide products in which there is at least one nucleic acid sequence difference, or nucleotide/nucleoside modification, between the terminal adapter sequences. Attaching adapters asymmetrically to polynucleotides (e.g., polynucleotide fragments) results in polynucleotides that have one or more adapter sequences on one end (e.g., one or more region or domain, e.g., a primer site) that are either not present or have a different nucleic acid sequence as compared to the adapter sequence on the other end. It is noted that an adapter that is termed an "asymmetric adapter" is not necessarily itself structurally asymmetric, nor does the mere act of attaching an asymmetric adapter to a polynucleotide fragment render it immediately asymmetric. Rather, an asymmetric adapter-attached polynucleotide, which has an identical asymmetric adapter at each end, produces replication products (or isolated single stranded polynucleotides) that are asymmetric with respect to the adapter sequences on opposite ends (e.g., after at least one round of amplification/primer extension).

Any convenient asymmetric adapter, or process for attaching adapters asymmetrically, may be employed in practicing the present invention. Exemplary asymmetric adapters are described in: U.S. Pat. Nos. 5,712,126 and 6,372,434; U.S. Patent Publications 2007/0128624 and 2007/0172839; and PCT publication WO/2009/032167; all of which are incorporated by reference herein in their entirety. In certain embodiments, the asymmetric adapters

employed are those described in U.S. patent application Ser. No. 12/432,080, filed on Apr. 29, 2009, incorporated herein by reference in its entirety.

"Complementary" or "substantially complementary" refers to the hybridization or base pairing or the formation of a duplex between nucleotides or nucleic acids, such as, for instance, between the two strands of a double stranded DNA molecule or between an oligonucleotide primer and a primer site on a single stranded nucleic acid. Complementary nucleotides are, generally, A and T (or A and U), or C and G. Two single stranded RNA or DNA molecules are said to be substantially complementary when the nucleotides of one strand, optimally aligned and compared and with appropriate nucleotide insertions or deletions, pair with at least about 80% of the nucleotides of the other strand, usually at least about 90% to 95%, and more preferably from about 98 to 100%. Alternatively, substantial complementarity exists when an RNA or DNA strand will hybridize under selective hybridization conditions to its complement. Typically, selective hybridization will occur when there is at least about 65% complementary over a stretch of at least 14 to 25 nucleotides, preferably at least about 75%, more preferably at least about 90% complementary. See, M. Kanehisa Nucleic Acids Res. 12:203 (1984), incorporated herein by reference.

"Duplex" means at least two oligonucleotides and/or polynucleotides that are fully or partially complementary undergo Watson-Crick type base pairing among all or most of their nucleotides so that a stable complex is formed. The terms "annealing" and "hybridization" are used interchangeably to mean the formation of a stable duplex. "Perfectly matched" in reference to a duplex means that the poly- or oligonucleotide strands making up the duplex form a double stranded structure with one another such that every nucleotide in each strand undergoes Watson-Crick base pairing with a nucleotide in the other strand. A stable duplex can include Watson-Crick base pairing and/or non-Watson-Crick base pairing between the strands of the duplex (where base pairing means the forming hydrogen bonds). In certain embodiments, a non-Watson-Crick base pair includes a nucleoside analog, such as deoxyinosine, 2, 6-diaminopurine, PNAs, LNA's and the like. In certain embodiments, a non-Watson-Crick base pair includes a "wobble base", such as deoxyinosine, 8-oxo-dA, 8-oxo-dG and the like, where by "wobble base" is meant a nucleic acid base that can base pair with a first nucleotide base in a complementary nucleic acid strand but that, when employed as a template strand for nucleic acid synthesis, leads to the incorporation of a second, different nucleotide base into the synthesizing strand (wobble bases are described in further detail below). A "mismatch" in a duplex between two oligonucleotides or polynucleotides means that a pair of nucleotides in the duplex fails to undergo Watson-Crick bonding.

"Genetic locus," "locus," or "locus of interest" in reference to a genome or target polynucleotide, means a contiguous sub-region or segment of the genome or target polynucleotide. As used herein, genetic locus, locus, or locus of interest may refer to the position of a nucleotide, a gene or a portion of a gene in a genome, including mitochondrial DNA or other non-chromosomal DNA (e.g., bacterial plasmid), or it may refer to any contiguous portion of genomic sequence whether or not it is within, or associated with, a gene. A genetic locus, locus, or locus of interest can be from a single nucleotide to a segment of a few hundred or a few thousand nucleotides in length or more. In general, a locus of interest will have a reference sequence associated with it (see description of "reference sequence" below).

"Kit" refers to any delivery system for delivering materials or reagents for carrying out a method of the invention. In the context of reaction assays, such delivery systems include systems that allow for the storage, transport, or delivery of reaction reagents (e.g., probes, enzymes, etc. in the appropriate containers) and/or supporting materials (e.g., buffers, written instructions for performing the assay etc.) from one location to another. For example, kits include one or more enclosures (e.g., boxes) containing the relevant reaction reagents and/or supporting materials. Such contents may be delivered to the intended recipient together or separately. For example, a first container may contain an enzyme for use in an assay, while a second container contains probes.

"Ligation" means to form a covalent bond or linkage between the termini of two or more nucleic acids, e.g. oligonucleotides and/or polynucleotides, in a template-driven reaction. The nature of the bond or linkage may vary widely and the ligation may be carried out enzymatically or chemically. As used herein, ligations are usually carried out enzymatically to form a phosphodiester linkage between a 5' carbon of a terminal nucleotide of one oligonucleotide with 3' carbon of another oligonucleotide. A variety of template-driven ligation reactions are described in the following references, which are incorporated by reference: Whiteley et al, U.S. Pat. No. 4,883,750; Letsinger et al, U.S. Pat. No. 5,476,930; Fung et al, U.S. Pat. No. 5,593,826; Kool, U.S. Pat. No. 5,426,180; Landegren et al, U.S. Pat. No. 5,871,921; Xu and Kool, Nucleic Acids Research, 27: 875-881 (1999); Higgins et al, Methods in Enzymology, 68: 50-71 (1979); Engler et al, The Enzymes, 15: 3-29 (1982); and Namsaraev, U.S. patent publication 2004/0110213.

"Multiplex Identifier" (MID) as used herein refers to a tag or combination of tags associated with a polynucleotide whose identity (e.g., the tag DNA sequence) can be used to differentiate polynucleotides in a sample. In certain embodiments, the MID on a polynucleotide is used to identify the source from which the polynucleotide is derived. For example, a nucleic acid sample may be a pool of polynucleotides derived from different sources, (e.g., polynucleotides derived from different individuals, different tissues or cells, or polynucleotides isolated at different times points), where the polynucleotides from each different source are tagged with a unique MID. As such, a MID provides a correlation between a polynucleotide and its source. In certain embodiments, MIDs are employed to uniquely tag each individual polynucleotide in a sample. Identification of the number of unique MIDs in a sample can provide a readout of how many individual polynucleotides are present in the sample (or from how many original polynucleotides a manipulated polynucleotide sample was derived; see, e.g., U.S. Pat. No. 7,537,897, issued on May 26, 2009, incorporated herein by reference in its entirety). MIDs can range in length from 2 to 100 nucleotide bases or more and may include multiple subunits, where each different MID has a distinct identity and/or order of subunits. Exemplary nucleic acid tags that find use as MIDs are described in U.S. Pat. No. 7,544,473, issued on Jun. 6, 2009, and titled "Nucleic Acid Analysis Using Sequence Tokens", as well as U.S. Pat. No. 7,393,665, issued on Jul. 1, 2008, and titled "Methods and Compositions for Tagging and Identifying Polynucleotides", both of which are incorporated herein by reference in their entirety for their description of nucleic acid tags and their use in identifying polynucleotides. In certain embodiments, a set of MIDs employed to tag a plurality of samples need not have any particular common property (e.g., Tm, length, base composition, etc.), as the methods described herein can

accommodate a wide variety of unique MID sets. It is emphasized here that MIDs need only be unique within a given experiment. Thus, the same MID may be used to tag a different sample being processed in a different experiment. In addition, in certain experiments, a user may use the same MID to tag a subset of different samples within the same experiment. For example, all samples derived from individuals having a specific phenotype may be tagged with the same MID, e.g., all samples derived from control (or wild-type) subjects can be tagged with a first MID while subjects having a disease condition can be tagged with a second MID (different than the first MID). As another example, it may be desirable to tag different samples derived from the same source with different MIDs (e.g., samples derived over time or derived from different sites within a tissue). Further, MIDs can be generated in a variety of different ways, e.g., by a combinatorial tagging approach in which one MID is attached by ligation and a second MID is attached by primer extension. Thus, MIDs can be designed and implemented in a variety of different ways to track polynucleotide fragments during processing and analysis, and thus no limitation in this regard is intended.

"Nucleoside" as used herein includes the natural nucleosides, including 2'-deoxy and 2'-hydroxyl forms, e.g. as described in Kornberg and Baker, DNA Replication, 2nd Ed. (Freeman, San Francisco, 1992). "Analogs" in reference to nucleosides includes synthetic nucleosides having modified base moieties and/or modified sugar moieties, e.g. described by Scheit, Nucleotide Analogs (John Wiley, New York, 1980); Uhlman and Peyman, Chemical Reviews, 90: 543-584 (1990), or the like, with the proviso that they are capable of specific hybridization. Such analogs include synthetic nucleosides designed to enhance binding properties, reduce complexity, increase specificity, and the like. Polynucleotides comprising analogs with enhanced hybridization or nuclease resistance properties are described in Uhlman and Peyman (cited above); Crooke et al, Exp. Opin. Ther. Patents, 6: 855-870 (1996); Mesmaeker et al, Current Opinion in Structual Biology, 5: 343-355 (1995); and the like. Exemplary types of polynucleotides that are capable of enhancing duplex stability include oligonucleotide N3'→P5' phosphoramidates (referred to herein as "amidates"), peptide nucleic acids (referred to herein as "PNAs"), oligo-2'-O-alkylribonucleotides, polynucleotides containing C-5 propynylpyrimidines, locked nucleic acids ("LNAs"), and like compounds. Such oligonucleotides are either available commercially or may be synthesized using methods described in the literature.

"Polymerase chain reaction," or "PCR," means a reaction for the in vitro amplification of specific DNA sequences by the simultaneous primer extension of complementary strands of DNA. In other words, PCR is a reaction for making multiple copies or replicates of a target nucleic acid flanked by primer sites, such reaction comprising one or more repetitions of the following steps: (i) denaturing the target nucleic acid, (ii) annealing primers to the primer sites, and (iii) extending the primers by a nucleic acid polymerase in the presence of nucleoside triphosphates. Usually, the reaction is cycled through different temperatures optimized for each step in a thermal cycler instrument. Particular temperatures, durations at each step, and rates of change between steps depend on many factors well-known to those of ordinary skill in the art, e.g. exemplified by the references: McPherson et al, editors, PCR: A Practical Approach and PCR2: A Practical Approach (IRL Press, Oxford, 1991 and 1995, respectively). For example, in a conventional PCR using Taq DNA polymerase, a double stranded target nucleic acid may be denatured at a temperature >90° C., primers annealed at a temperature in the range 50-75° C., and primers extended at a temperature in the range 72-78° C. The term "PCR" encompasses derivative forms of the reaction, including but not limited to, RT-PCR, real-time PCR, nested PCR, quantitative PCR, multiplexed PCR, and the like. Reaction volumes range from a few hundred nanoliters, e.g. 200 nL, to a few hundred e.g. 200 μL. "Reverse transcription PCR," or "RT-PCR," means a PCR that is preceded by a reverse transcription reaction that converts a target RNA to a complementary single stranded DNA, which is then amplified, e.g. Tecott et al, U.S. Pat. No. 5,168,038, which patent is incorporated herein by reference. "Real-time PCR" means a PCR for which the amount of reaction product, i.e. amplicon, is monitored as the reaction proceeds. There are many forms of real-time PCR that differ mainly in the detection chemistries used for monitoring the reaction product, e.g. Gelfand et al, U.S. Pat. No. 5,210,015 ("TAQMAN™"); Wittwer et al, U.S. Pat. Nos. 6,174,670 and 6,569,627 (intercalating dyes); Tyagi et al, U.S. Pat. No. 5,925,517 (molecular beacons); which patents are incorporated herein by reference. Detection chemistries for real-time PCR are reviewed in Mackay et al, Nucleic Acids Research, 30: 1292-1305 (2002), which is also incorporated herein by reference. "Nested PCR" means a two-stage PCR wherein the amplicon of a first PCR becomes the sample for a second PCR using a new set of primers, at least one of which binds to an interior location of the first amplicon. As used herein, "initial primers" in reference to a nested amplification reaction mean the primers used to generate a first amplicon, and "secondary primers" mean the one or more primers used to generate a second, or nested, amplicon. "Multiplexed PCR" means a PCR wherein multiple target sequences (or a single target sequence and one or more reference sequences) are simultaneously carried out in the same reaction mixture, e.g. Bernard et al, Anal. Biochem., 273: 221-228 (1999)(two-color real-time PCR). Usually, distinct sets of primers are employed for each sequence being amplified.

"Quantitative PCR" means a PCR designed to measure the abundance of one or more specific target sequences in a sample or specimen. Quantitative PCR includes both absolute quantitation and relative quantitation of such target sequences. Quantitative measurements are made using one or more reference sequences that may be assayed separately or together with a target sequence. The reference sequence may be endogenous or exogenous to a sample or specimen, and in the latter case, may comprise one or more competitor templates. Typical endogenous reference sequences include segments of transcripts of the following genes: β-actin, GAPDH, β_2-microglobulin, ribosomal RNA, and the like. Techniques for quantitative PCR are well-known to those of ordinary skill in the art, as exemplified in the following references that are incorporated by reference: Freeman et al, Biotechniques, 26: 112-126 (1999); Becker-Andre et al, Nucleic Acids Research, 17: 9437-9447 (1989); Zimmerman et al, Biotechniques, 21: 268-279 (1996); Diviacco et al, Gene, 122: 3013-3020 (1992); Becker-Andre et al, Nucleic Acids Research, 17: 9437-9446 (1989); and the like.

"Polynucleotide" or "oligonucleotide" is used interchangeably and each means a linear polymer of nucleotide monomers. Monomers making up polynucleotides and oligonucleotides are capable of specifically binding to a natural polynucleotide by way of a regular pattern of monomer-to-monomer interactions, such as Watson-Crick type of base pairing, base stacking, Hoogsteen or reverse Hoogsteen types of base pairing, wobble base pairing, or the like. As

described in detail below, by "wobble base" is meant a nucleic acid base that can base pair with a first nucleotide base in a complementary nucleic acid strand but that, when employed as a template strand for nucleic acid synthesis, leads to the incorporation of a second, different nucleotide base into the synthesizing strand. Such monomers and their internucleosidic linkages may be naturally occurring or may be analogs thereof, e.g. naturally occurring or non-naturally occurring analogs. Non-naturally occurring analogs may include peptide nucleic acids (PNAs, e.g., as described in U.S. Pat. No. 5,539,082, incorporated herein by reference), locked nucleic acids (LNAs, e.g., as described in U.S. Pat. No. 6,670,461, incorporated herein by reference), phosphorothioate internucleosidic linkages, bases containing linking groups permitting the attachment of labels, such as fluorophores, or haptens, and the like. Whenever the use of an oligonucleotide or polynucleotide requires enzymatic processing, such as extension by a polymerase, ligation by a ligase, or the like, one of ordinary skill would understand that oligonucleotides or polynucleotides in those instances would not contain certain analogs of internucleosidic linkages, sugar moieties, or bases at any or some positions. Polynucleotides typically range in size from a few monomeric units, e.g. 5-40, when they are usually referred to as "oligonucleotides," to several thousand monomeric units. Whenever a polynucleotide or oligonucleotide is represented by a sequence of letters (upper or lower case), such as "ATGCCTG," it will be understood that the nucleotides are in 5'→3' order from left to right and that "A" denotes deoxyadenosine, "C" denotes deoxycytidine, "G" denotes deoxyguanosine, and "T" denotes thymidine, "I" denotes deoxyinosine, "U" denotes uridine, unless otherwise indicated or obvious from context. Unless otherwise noted the terminology and atom numbering conventions will follow those disclosed in Strachan and Read, Human Molecular Genetics 2 (Wiley-Liss, New York, 1999). Usually polynucleotides comprise the four natural nucleosides (e.g. deoxyadenosine, deoxycytidine, deoxyguanosine, deoxythymidine for DNA or their ribose counterparts for RNA) linked by phosphodiester linkages; however, they may also comprise non-natural nucleotide analogs, e.g. including modified bases, sugars, or internucleosidic linkages. It is clear to those skilled in the art that where an enzyme has specific oligonucleotide or polynucleotide substrate requirements for activity, e.g. single stranded DNA, RNA/DNA duplex, or the like, then selection of appropriate composition for the oligonucleotide or polynucleotide substrates is well within the knowledge of one of ordinary skill, especially with guidance from treatises, such as Sambrook et al, Molecular Cloning, Second Edition (Cold Spring Harbor Laboratory, New York, 1989), and like references.

"Primer" means an oligonucleotide, either natural or synthetic, that is capable, upon forming a duplex with a polynucleotide template, of acting as a point of initiation of nucleic acid synthesis and being extended from its 3' end along the template so that an extended duplex is formed. The sequence of nucleotides added during the extension process is determined by the sequence of the template polynucleotide. Usually primers are extended by a DNA polymerase. Primers are generally of a length compatible with their use in synthesis of primer extension products, and are usually are in the range of between 8 to 100 nucleotides in length, such as 10 to 75, 15 to 60, 15 to 40, 18 to 30, 20 to 40, 21 to 50, 22 to 45, 25 to 40, and so on, more typically in the range of between 18-40, 20-35, 21-30 nucleotides long, and any length between the stated ranges. Typical primers can be in the range of between 10-50 nucleotides long, such as 15-45, 18-40, 20-30, 21-25 and so on, and any length between the stated ranges. In some embodiments, the primers are usually not more than about 10, 12, 15, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 35, 40, 45, 50, 55, 60, 65, or 70 nucleotides in length.

Primers are usually single-stranded for maximum efficiency in amplification, but may alternatively be double-stranded. If double-stranded, the primer is usually first treated to separate its strands before being used to prepare extension products. This denaturation step is typically affected by heat, but may alternatively be carried out using alkali, followed by neutralization. Thus, a "primer" is complementary to a template, and complexes by hydrogen bonding or hybridization with the template to give a primer/template complex for initiation of synthesis by a polymerase, which is extended by the addition of covalently bonded bases linked at its 3' end complementary to the template in the process of DNA synthesis.

A "primer pair" as used herein refers to first and second primers having nucleic acid sequence suitable for nucleic acid-based amplification of a target nucleic acid. Such primer pairs generally include a first primer having a sequence that is the same or similar to that of a first portion of a target nucleic acid, and a second primer having a sequence that is complementary to a second portion of a target nucleic acid to provide for amplification of the target nucleic acid or a fragment thereof. Reference to "first" and "second" primers herein is arbitrary, unless specifically indicated otherwise. For example, the first primer can be designed as a "forward primer" (which initiates nucleic acid synthesis from a 5' end of the target nucleic acid) or as a "reverse primer" (which initiates nucleic acid synthesis from a 5' end of the extension product produced from synthesis initiated from the forward primer). Likewise, the second primer can be designed as a forward primer or a reverse primer.

"Primer site" (e.g., a sequencing primer site, and amplification primer site, etc.) as used herein refers to a domain in a polynucleotide that includes the sequence of a primer (e.g., a sequencing primer) and/or the complementary sequence of a primer. When present in single stranded form (e.g., in a single stranded polynucleotide), a primer site can be either the identical sequence of a primer or the complementary sequence of a primer. When present in double stranded form, a primer site contains the sequence of a primer hybridized to the complementary sequence of the primer. Thus, a primer site is a region of a polynucleotide that is either identical to or complementary to the sequence of a primer (when in a single stranded form) or a double stranded region formed between a primer sequence and its complement. Primer sites may be present in an adapter attached to a polynucleotide. The specific orientation of a primer site can be inferred by those of ordinary skill in the art from the structural features of the relevant polynucleotide and/or context in which it is used.

"Readout" means a parameter, or parameters, which are measured and/or detected that can be converted to a number or value. In some contexts, readout may refer to an actual numerical representation of such collected or recorded data. For example, a readout of fluorescent intensity signals from a microarray is the address and fluorescence intensity of a signal being generated at each hybridization site of the microarray; thus, such a readout may be registered or stored in various ways, for example, as an image of the microarray, as a table of numbers, or the like.

"Reflex site", "reflex sequence" and equivalents are used to indicate sequences in a polynucleotide that are employed

to move a domain intramolecularly from its initial location to a different location in the polynucleotide. The sequence of a reflex site can be added to a polynucleotide of interest (e.g., present in an adapter ligated to the polynucleotide), be based on a sequence naturally present within the polynucleotide of interest (e.g., a genomic sequence in the polynucleotide), or a combination of both. The reflex sequence is chosen so as to be distinct from other sequences in the polynucleotide (i.e., with little sequence homology to other sequences likely to be present in the polynucleotide, e.g., genomic or sub-genomic sequences to be processed). As such, a reflex sequence should be selected so as to not hybridize to any sequence except its complement under the conditions employed in the reflex processes herein described. As described later in this application, the complement to the reflex sequence is inserted on the same strand of the polynucleotide (e.g., the same strand of a double-stranded polynucleotide or on the same single stranded polynucleotide) in a particular location so as to facilitate an intramolecular binding event on such particular strand. Reflex sequences employed in the reflex process described herein can thus have a wide range of lengths and sequences. Reflex sequences may range from 5 to 200 nucleotide bases in length.

"Solid support", "support", and "solid phase support" are used interchangeably and refer to a material or group of materials having a rigid or semi-rigid surface or surfaces. In many embodiments, at least one surface of the solid support will be substantially flat, although in some embodiments it may be desirable to physically separate synthesis regions for different compounds with, for example, wells, raised regions, pins, etched trenches, or the like. According to other embodiments, the solid support(s) will take the form of beads, resins, gels, microspheres, or other geometric configurations. Microarrays usually comprise at least one planar solid phase support, such as a glass microscope slide.

"Specific" or "specificity" in reference to the binding of one molecule to another molecule, such as a labeled target sequence for a probe, means the recognition, contact, and formation of a stable complex between the two molecules, together with substantially less recognition, contact, or complex formation of that molecule with other molecules. In one aspect, "specific" in reference to the binding of a first molecule to a second molecule means that to the extent the first molecule recognizes and forms a complex with another molecule in a reaction or sample, it forms the largest number of the complexes with the second molecule. Preferably, this largest number is at least fifty percent. Generally, molecules involved in a specific binding event have areas on their surfaces or in cavities giving rise to specific recognition between the molecules binding to each other. Examples of specific binding include antibody-antigen interactions, enzyme-substrate interactions, formation of duplexes or triplexes among polynucleotides and/or oligonucleotides, biotin-avidin or biotin-streptavidin interactions, receptor-ligand interactions, and the like. As used herein, "contact" in reference to specificity or specific binding means two molecules are close enough that weak noncovalent chemical interactions, such as Van der Waal forces, hydrogen bonding, base-stacking interactions, ionic and hydrophobic interactions, and the like, dominate the interaction of the molecules.

As used herein, the term "T_m" is used in reference to the "melting temperature." The melting temperature is the temperature (e.g., as measured in ° C.) at which a population of double-stranded nucleic acid molecules becomes half dissociated into single strands. Several equations for calculating the T. of nucleic acids are known in the art (see e.g., Anderson and Young, Quantitative Filter Hybridization, in Nucleic Acid Hybridization (1985). Other references (e.g., Allawi, H. T. & SantaLucia, J., Jr., Biochemistry 36, 10581-94 (1997)) include alternative methods of computation which take structural and environmental, as well as sequence characteristics into account for the calculation of T_m.

"Sample" means a quantity of material from a biological, environmental, medical, or patient source in which detection, measurement, or labeling of target nucleic acids is sought. On the one hand it is meant to include a specimen or culture (e.g., microbiological cultures). On the other hand, it is meant to include both biological and environmental samples. A sample may include a specimen of synthetic origin. Biological samples may be animal, including human, fluid, solid (e.g., stool) or tissue, as well as liquid and solid food and feed products and ingredients such as dairy items, vegetables, meat and meat by-products, and waste. Biological samples may include materials taken from a patient including, but not limited to cultures, blood, saliva, cerebral spinal fluid, pleural fluid, milk, lymph, sputum, semen, needle aspirates, and the like. Biological samples may be obtained from all of the various families of domestic animals, as well as feral or wild animals, including, but not limited to, such animals as ungulates, bear, fish, rodents, etc. Environmental samples include environmental material such as surface matter, soil, water and industrial samples, as well as samples obtained from food and dairy processing instruments, apparatus, equipment, utensils, disposable and non-disposable items. These examples are not to be construed as limiting the sample types applicable to the present invention.

The terms "upstream" and "downstream" in describing nucleic acid molecule orientation and/or polymerization are used herein as understood by one of skill in the art. As such, "downstream" generally means proceeding in the 5' to 3' direction, i.e., the direction in which a nucleotide polymerase normally extends a sequence, and "upstream" generally means the converse. For example, a first primer that hybridizes "upstream" of a second primer on the same target nucleic acid molecule is located on the 5' side of the second primer (and thus nucleic acid polymerization from the first primer proceeds towards the second primer).

It is further noted that the claims may be drafted to exclude any optional element. As such, this statement is intended to serve as antecedent basis for use of such exclusive terminology as "solely", "only" and the like in connection with the recitation of claim elements, or the use of a "negative" limitation.

DETAILED DESCRIPTION OF THE INVENTION

The invention is drawn to compositions and methods for intramolecular nucleic acid rearrangement that find use in various applications of genetic analysis, including sequencing, as well as general molecular biological manipulations of polynucleotide structures.

Before the present invention is described, it is to be understood that this invention is not limited to particular embodiments described, as such may, of course, vary. It is also to be understood that the terminology used herein is for the purpose of describing particular embodiments only, and is not intended to be limiting, since the scope of the present invention will be limited only by the appended claims.

Where a range of values is provided, it is understood that each intervening value, to the tenth of the unit of the lower limit unless the context clearly dictates otherwise, between the upper and lower limits of that range is also specifically

disclosed. Each smaller range between any stated value or intervening value in a stated range and any other stated or intervening value in that stated range is encompassed within the invention. The upper and lower limits of these smaller ranges may independently be included or excluded in the range, and each range where either, neither or both limits are included in the smaller ranges is also encompassed within the invention, subject to any specifically excluded limit in the stated range. Where the stated range includes one or both of the limits, ranges excluding either or both of those included limits are also included in the invention.

Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. Although any methods and materials similar or equivalent to those described herein can be used in the practice or testing of the present invention, some potential and preferred methods and materials are now described. All publications mentioned herein are incorporated herein by reference to disclose and describe the methods and/or materials in connection with which the publications are cited. It is understood that the present disclosure supersedes any disclosure of an incorporated publication to the extent there is a contradiction.

It must be noted that as used herein and in the appended claims, the singular forms "a", "an", and "the" include plural referents unless the context clearly dictates otherwise. Thus, for example, reference to "a nucleic acid" includes a plurality of such nucleic acids and reference to "the compound" includes reference to one or more compounds and equivalents thereof known to those skilled in the art, and so forth.

The practice of the present invention may employ, unless otherwise indicated, conventional techniques and descriptions of organic chemistry, polymer technology, molecular biology (including recombinant techniques), cell biology, biochemistry, and immunology, which are within the skill of the art. Such conventional techniques include polymer array synthesis, hybridization, ligation, and detection of hybridization using a label. Specific illustrations of suitable techniques can be had by reference to the example herein below. However, other equivalent conventional procedures can, of course, also be used. Such conventional techniques and descriptions can be found in standard laboratory manuals such as *Genome Analysis: A Laboratory Manual Series* (Vols. I-IV), *Using Antibodies: A Laboratory Manual, Cells: A Laboratory Manual, PCR Primer: A Laboratory Manual, and Molecular Cloning: A Laboratory Manual* (all from Cold Spring Harbor Laboratory Press), Stryer, L. (1995) *Biochemistry* (4th Ed.) Freeman, New York, Gait, "*Oligonucleotide Synthesis: A Practical Approach*" 1984, IRL Press, London, Nelson and Cox (2000), Lehninger, A., *Principles of Biochemistry* 3rd Ed., W. H. Freeman Pub., New York, N.Y. and Berg et al. (2002) *Biochemistry,* 5th Ed., W. H. Freeman Pub., New York, N.Y., all of which are herein incorporated in their entirety by reference for all purposes.

The publications discussed herein are provided solely for their disclosure prior to the filing date of the present application. Nothing herein is to be construed as an admission that the present invention is not entitled to antedate such publication by virtue of prior invention. Further, the dates of publication provided may be different from the actual publication dates which may need to be independently confirmed.

As summarized above, aspects of the present invention are drawn to the use of a 'reflex' sequence present in a polynucleotide (e.g., in an adapter structure of the polynucleotide, in a genomic region of the polynucleotide, or a combination of both) to move a domain of the polynucleotide intra-molecularly from a first location to a second location. The reflex process described herein finds use in any number of applications, e.g., placing functional elements of a polynucleotide (e.g., sequencing primer sites and/or MID tags) into proximity to a desired sub-region of interest.

Nucleic Acids

The reflex process (as described in detail below) can be employed for the manipulation and analysis of nucleic acid sequences of interest from virtually any nucleic acid source, including but not limited to genomic DNA, complementary DNA (cDNA), RNA (e.g., messenger RNA, ribosomal RNA, short interfering RNA, microRNA, etc.), plasmid DNA, mitochondrial DNA, synthetic DNA, etc. Furthermore, any organism, organic material or nucleic acid-containing substance can be used as a source of nucleic acids to be processed in accordance with the present invention including, but not limited to, plants, animals (e.g., reptiles, mammals, insects, worms, fish, etc.), tissue samples, bacteria, fungi (e.g., yeast), phage, viruses, cadaveric tissue, archaeological/ancient samples, etc. In certain embodiments, the nucleic acids in the nucleic acid sample are derived from a mammal, where in certain embodiments the mammal is a human.

In certain embodiments, the nucleic acid sequences are enriched prior to the reflex sequence process. By enriched is meant that the nucleic acid is subjected to a process that reduces the complexity of the nucleic acids, generally by increasing the relative concentration of particular nucleic acid species in the sample (e.g., having a specific locus of interest, including a specific nucleic acid sequence, lacking a locus or sequence, being within a specific size range, etc.). There are a wide variety of ways to enrich nucleic acids having a specific characteristic(s) or sequence, and as such any convenient method to accomplish this may be employed. The enrichment (or complexity reduction) can take place at any of a number of steps in the process, and will be determined by the desires of the user. For example, enrichment can take place in individual parental samples (e.g., untagged nucleic acids prior to adaptor ligation) or in multiplexed samples (e.g., nucleic acids tagged with primer sites, MID and/or reflex sequences and pooled; MID are described in further detail below).

In certain embodiments, nucleic acids in the nucleic acid sample are amplified prior to analysis. In certain of these embodiments, the amplification reaction also serves to enrich a starting nucleic acid sample for a sequence or locus of interest. For example, a starting nucleic acid sample can be subjected to a polymerase chain reaction (PCR) that amplifies one or more region of interest. In certain embodiments, the amplification reaction is an exponential amplification reaction, whereas in certain other embodiments, the amplification reaction is a linear amplification reaction. Any convenient method for performing amplification reactions on a starting nucleic acid sample can be used in practicing the subject invention. In certain embodiments, the nucleic acid polymerase employed in the amplification reaction is a polymerase that has proofreading capability (e.g., phi29 DNA Polymerase, *Thermococcus litoralis* DNA polymerase, *Pyrococcus furiosus* DNA polymerase, etc.).

In certain embodiments, the nucleic acid sample being analyzed is derived from a single source (e.g., a single organism, virus, tissue, cell, subject, etc.), whereas in other embodiments, the nucleic acid sample is a pool of nucleic acids extracted from a plurality of sources (e.g., a pool of nucleic acids from a plurality of organisms, tissues, cells, subjects, etc.), where by "plurality" is meant two or more. As

such, in certain embodiments, a nucleic acid sample can contain nucleic acids from 2 or more sources, 3 or more sources, 5 or more sources, 10 or more sources, 50 or more sources, 100 or more sources, 500 or more sources, 1000 or more sources, 5000 or more sources, up to and including about 10,000 or more sources.

In certain embodiments, nucleic acid fragments that are to be pooled with nucleic acid fragments derived from a plurality of sources (e.g., a plurality of organisms, tissues, cells, subjects, etc.), where by "plurality" is meant two or more. In such embodiments, the nucleic acids derived from each source includes a multiplex identifier (MID) such that the source from which the each tagged nucleic acid fragment was derived can be determined. In such embodiments, each nucleic acid sample source is correlated with a unique MID, where by unique MID is meant that each different MID employed can be differentiated from every other MID employed by virtue of at least one characteristic, e.g., the nucleic acid sequence of the MID. Any type of MID can be used, including but not limited to those described in co-pending U.S. patent application Ser. No. 11/656,746, filed on Jan. 22, 2007, and titled "Nucleic Acid Analysis Using Sequence Tokens", as well as U.S. Pat. No. 7,393,665, issued on Jul. 1, 2008, and titled "Methods and Compositions for Tagging and Identifying Polynucleotides", both of which are incorporated herein by reference in their entirety for their description of nucleic acid tags and their use in identifying polynucleotides. In certain embodiments, a set of MIDs employed to tag a plurality of samples need not have any particular common property (e.g., T_m, length, base composition, etc.), as the asymmetric tagging methods (and many tag readout methods, including but not limited to sequencing of the tag or measuring the length of the tag) can accommodate a wide variety of unique MID sets.

In certain embodiments, each individual polynucleotide (e.g., double-stranded or single-stranded, as appropriate to the methodological details employed) in a sample to be analyzed is tagged with a unique MID so that the fate of each polynucleotide can be tracked in subsequent processes (where, as noted above, unique MID is meant to indicate that each different MID employed can be differentiated from every other MID employed by virtue of at least one characteristic, e.g., the nucleic acid sequence of the MID). For example (and as described below), having each nucleic acid tagged with a unique MID allows analysis of the sequence of each individual nucleic acid using the reflex sequence methods described herein. This allows the linkage of sequence information for large nucleic acid fragments that cannot be sequenced in a single sequencing run.

Reflex Sequence Process

As summarized above, aspects of the present invention include methods and compositions for moving a domain in a polynucleotide from a first location to a second location in the polynucleotide. An exemplary embodiment is shown in FIG. **1**A.

FIG. **1**A shows a single stranded polynucleotide 100 comprising, in a 5' to 3' orientation, a first domain (102; the domain to be moved); a reflex sequence 104; a nucleic acid sequence 106 having a site distal to the first domain (Site A), and a complement of the reflex sequence 108 (positioned at the 3' terminus of the polynucleotide). The steps of the reflex method described below will move the first domain into closer proximity to Site A. It is noted here that the prime designation in FIG. **1**A denotes a complementary sequence of a domain. For example, First Domain' is the complement of the First Domain.

In Step 1, the reflex sequence and its complement in the polynucleotide are annealed intramolecularly to form polynucleotide structure 112, with the polynucleotide folding back on itself and hybridizing to form a region of complementarity (i.e., double stranded reflex/reflex' region). In this configuration, the 3' end of the complement of the reflex sequence can serve as a nucleic acid synthesis priming site. Nucleic acid synthesis from this site is then performed in extension Step 2 producing a complement of the first domain at the 3' end of the nucleic acid extension (shown in polynucleotide 114; extension is indicated with dotted arrow labeled "extend").

Denaturation of polynucleotide 114 (e.g., by heat) generates linear single stranded polynucleotide 116. As shown in FIG. **1**, resultant polynucleotide 116 contains a complement of the first domain at a position proximal to Site A (i.e., separated by only the complement of the reflex sequence). This resultant polynucleotide may be used for any subsequent analysis or processing steps as desired by the user (e.g., sequencing, as a template for amplification (linear, PCR, etc.), sequence specific extraction, etc.).

In alternative embodiments, the first domain and reflex sequence are removed from the 5' end of the double-stranded region of polynucleotide 114 (shown in polynucleotide 118; removal is shown in the dotted arrow labeled "remove"). Removal of this region may be accomplished by any convenient method, including, but not limited to, treatment (under appropriate incubation conditions) of polynucleotide structure 114 with T7 exonuclease or by treatment with Lambda exonuclease; the Lambda exonuclease can be employed so long as the 5' end of the polynucleotide is phosphorylated. If the region is removed enzymatically, resultant polynucleotide 118 is used in place of polynucleotide 116 in subsequent steps (e.g., copying to reverse polarity).

In certain embodiments, polynucleotide 116 or 118 is used as a template to produce a double stranded polynucleotide, for example by performing a nucleic acid synthesis reaction with a primer that primes in the complement of the first domain. This step is sometimes referred to as copying to reverse polarity of a single stranded polynucleotide, and in some instances, the double-stranded intermediate product of this copying is not shown (see, e.g., FIG. **3**). For example, copying to reverse the polarity of polynucleotide 116 results in single-stranded polynucleotide 120 having, in a 5' to 3' orientation, the first domain (122); the reflex sequence (124); the complement of polynucleotide 106 (oriented with the complement of Site A (Site A'; 126) proximal to the reflex sequence); the complement of the reflex sequence (128); and the complement of the first domain (130).

In certain embodiments, the first domain in the polynucleotide comprises one or more elements that find use in one or more subsequent processing or analysis steps. Such sequences include, but are not limited to, restriction enzyme sites, PCR primer sites, linear amplification primer sites, reverse transcription primer sites, RNA polymerase promoter sites (such as for T7, T3 or SP6 RNA polymerase), MID tags, sequencing primer sites, etc. Any convenient element can be included in the first domain and, in certain embodiments, is determined by the desires of the user of the methods described herein.

As an exemplary embodiment, suppose we want to sequence a specific polynucleotide region from multiple genomes in a pooled sample where the polynucleotide region is too long to sequence in a single reaction. For example, sequencing a polynucleotide region that is 2 kilobases or more in length using Roche 454 (Branford, Conn.)

technology, in which the length of a single sequencing run is about 400 bases. In this scenario, we can design a set of left hand primers (A_n) and right hand primers (B_n) specific for the polynucleotide region that are positioned in such a way that we can obtain direct sequences of all parts of the insert, as shown in FIG. **1**B. Note that the polynucleotide shown in FIG. **1**B (140) has a domain (142) containing a primer site and an MID denoting from which original sample(s) the polynucleotide is derived. Site 142 thus represents an example of a First Domain site such identified as 122 in the FIG. **1**A. The polynucleotide also includes a reflex site (144), which can be part of the polynucleotide region itself (e.g., a genomic sequence), added in a ligated adapter domain along with the primer site and the MID (an artificial sequence), or a combination of both (a sequence spanning the adapter/polynucleotide junction).

It is noted here that polynucleotide 140 can be categorized as a precursor to polynucleotide 100 in FIG. **1**A, as it does not include a 3' reflex sequence complementary to the reflex site (domain 108 in FIG. **1**A). As detailed below, polynucleotide 140 can be converted to a polynucleotide having the structural configuration of polynucleotide 100, a polynucleotide suitable as a substrate for the reflex process described herein (e.g., by primer extension using a B_n primer and reversal of polarity).

In an exemplary embodiment, each A_n-B_n primer pair defines a nucleic acid region that is approximately 400 bases in length or less. This size range is within the single-sequencing run read length of the current Roche 454 sequencing platform; a different size range for the defined nucleic acid region may be utilized for a different sequencing platform. Thus, each product from each reflex process can be sequenced in a single run. It is noted here that primer pairs as shown in FIG. **1**B can be used to define regions 1 to 5 shown in FIG. **3** (described in further detail below).

In certain embodiments, to obtain the first part of the sequence of the polynucleotide region (i.e., in the original structure, that part of the polynucleotide closest to the first domain), we only need a right hand primer (e.g., B_0) and we do not need to transfer the MID as it is within reach of this sequencing primer (i.e., the MID is within 400 bases of sequencing primer B_0). All other B_n primers have the reflex sequence added to their 5' ends ("R" element shown on B primers) so that they read 5' reflex-B_n. However, in certain embodiments, the B_0 primer does include the reflex sequence and is used in the reflex process (along with a corresponding A_0 primer) as detailed below.

As described above, we obtain a single stranded polynucleotide having, in the 5' to 3' orientation, a primer site (e.g., for Roche 454 sequencing), an MID, a reflex sequence and the polynucleotide to be sequenced. Numerous methods for obtaining single-stranded polynucleotides of interest have been described and are known in the art, including in U.S. Pat. No. 7,217,522, issued on May 15, 2007; U.S. patent application Ser. No. 11/377,462, filed on Mar. 16, 2006; and U.S. patent application Ser. No. 12/432,080, filed on Apr. 29, 2009; each of which is incorporated by reference herein in their entirety. For example, a single stranded product can be produced using linear amplification with a primer specific for the primer site of the template. In certain embodiments, the primer includes a binding moiety to facilitate isolation of the single stranded nucleic acid of interest, e.g., to immobilize the top strand on a binding partner of the binding moiety immobilized on a solid support. Removal of a hybridized, non-biotinylated strand by denaturation using heat or high pH (or any other convenient method) serves to isolate the biotinylated strand. Binding moieties and their corresponding binding partners are sometimes referred to herein as binding partner pairs. Any convenient binding partner pairs may be used, including but not limited to biotin/avidin (or streptavidin), antigen/antibody pairs, etc.

It is noted here that while the figures and description of the reflex process provided herein depict manipulations with regard to a single stranded polynucleotide, it is not necessarily required that the single stranded polynucleotide described or depicted in the figures be present in the sample in an isolated form (i.e., isolated from its complementary strand). In other words, double stranded polynucleotides may be used where only one strand is described/depicted, which will generally be determined by the user.

The implementation of a single strand isolation step using the methods described above or variations thereof (or any other convenient single strand isolation step) will generally be based on the desires of the user. One example of isolating single stranded polynucleotides is shown in FIG. **2**. In this Figure, a starting double stranded template (with 5' to 3' orientation shown as an arrow) is denatured and primed with a biotinylated synthesis primer specific for the primer site. After extension of the primer (i.e., nucleic acid synthesis), the sample is contacted with a solid support having streptavidin bound to it. The biotin moiety (i.e., the binding partner of streptavidin) on the extended strands will bind to the solid-phase streptavidin. Denaturation and washing is then performed to remove all non-biotinylated polynucleotide strands. If desired, the bound polynucleotide, which can be used in subsequent reflex process steps (e.g., as a template for B_n primer extension reactions), may be eluted from the streptavidin support. Alternatively, the bound polynucleotide may be employed in subsequent steps of the desired process while still bound to the solid support (e.g., in solid phase extension reactions using B_n primers). This process, with minor variations depending on the template being used and the identity of the desired single stranded polynucleotide, may be employed at any of a number of steps in which a single stranded product is to be isolated. It is noted that in certain embodiments, substrate bound biotinylated polynucleotide can be used to produce and isolate non-biotinylated single stranded products (i.e., by eluting the non-biotinylated products while leaving the biotinylated templates bound to the streptavidin on the solid support). Thus, the specifics of how binding partners are used to isolate single stranded polynucleotides of interest will vary depending on experimental design parameters.

Additional single-stranded isolation/production methods include asymmetric PCR, strand-specific enzymatic degradation, and the use of in-vitro transcription followed by reverse transcriptase (IVT-RT) with subsequent destruction of the RNA strand. As noted above, any convenient single stranded production/isolation method may be employed.

To the single stranded polynucleotide shown in FIG. **1**B we anneal one of the B_n primers having the appended reflex sequence, denoted with a capital "R" (e.g., B_1) and extend the primer under nucleic acid synthesis conditions to produce a copy of the polynucleotide that has a reflex sequence at its 5' end. A single stranded copy of this polynucleotide is then produced to reverse polarity using a primer specific for the primer site in the first domain' (complement of the first domain 102). The resulting nucleic acid has structure 100 shown in FIG. **1**A, where the first domain 102 includes the primer site and the MID. Site A (110) in FIG. **1** is determined by the specificity of the 5' reflex-B_n primer used.

The reflex process (e.g., as shown in FIG. **1**) is then performed to produce a product in which the primer site and

the MID are now in close proximity to the desired site (or region of interest (ROI)) within the original polynucleotide (i.e., the site defined by the primer used, e.g., B_1). The resulting polynucleotide can be used in subsequent analyses as desired by the user (e.g., Roche 454 sequencing technology).

It is noted here that, while not shown in FIGS. **1**A and **1**B, any convenient method for adding adapters to a polynucleotide to be processed as described herein may be used in the practice of the reflex process (adapters containing, e.g., primer sites, polymerase sites, MIDs, restriction enzyme sites, and reflex sequences). For example, adapters can be added at a particular position by ligation. For double stranded polynucleotides, an adapter can be configured to be ligated to a particular restriction enzyme cut site. Where a single stranded polynucleotide is employed, a double stranded adapter construct that possesses an overhang configured to bind to the end of the single-stranded polynucleotide can be used. For example, in the latter case, the end of a single stranded polynucleotide can be modified to include specific nucleotide bases that are complementary to the overhang in the double stranded adaptor using terminal transferase and specific nucleotides. In other embodiments, PCR or linear amplification methods using adapter-conjugated primers is employed to add an adapter at a site of interest. Again, any convenient method for producing a starting polynucleotide may be employed in practicing the methods of the subject invention.

In certain embodiments, the nucleic acid may be sequenced directly using a sequencing primer specific for the primer site. This sequencing reaction will read through the MID and desired site in the insert.

In certain embodiments, the polynucleotide may be isolated (or fractionated) using an appropriate A_n primer (e.g., when using B_1 as the first primer, primer A_1 can be used). In certain embodiments, the A_n primed polynucleotide is subjected to nucleic acid synthesis conditions to produce a copy of the fragment produced in the reflex process. In certain of these embodiments, the A_n primer has appended on its 5' end a primer site that can be used in subsequent steps, including sequencing reactions. Providing a primer site in the A_n primer allows amplifying and/or sequencing from both ends of the resultant fragment: from the primer site in the first domain 102 and the primer site in the A_n primer (not shown in FIG. **1**B). Because of the position of the primer sites and their distance apart (i.e., less than one sequencing run apart), sequencing from both ends will usually capture the sequence of the desired site (or ROI) and the sequence of the MID, which can be used for subsequent bioinformatic analyses, e.g., to positively identify the sample of origin. It is noted here that while sequencing in both directions is possible, it is not necessary, as sequencing from either primer site alone will capture the sequence of the ROI as well as its corresponding MID sequence.

Note that in certain embodiments, the first fragment obtained by amplification/extension from primer B_0 directly, the polarity of the ROI in the resulting fragment is reversed as compared to the ROI in fragments obtained by primers B_1-B_n. This is because the B_0-generated fragment, unlike the B_1-B_n generated fragments, has not been subjected to a reflex process which reverses the orientation of the ROI sequence with respect to the first domain/reflex sequence (as described above). Therefore, the B_0 primer may have appended to it a primer site (e.g., at its 5' end) that can be used for subsequent amplification and/or sequencing reactions (e.g., in Roche 454 sequencing system) rather than a reflex sequence as with primers B_1-B_n. However, in certain embodiments, as noted above, the reflex process may be used with a corresponding B_0-A_0 primer pair as described above, i.e., using a B_0 primer having a 5' reflex sequence and a corresponding A_0 primer with its corresponding 5' adapter domain (e.g., a primer site).

It is noted here that because the particular sections of sequence to be analyzed are defined by the A_n-B_n primer pairs (as shown and described above), a much higher sequence specificity is achieved as compared to using previous extraction methods that employ only a single oligo binding event (e.g., using probes on a microarray).

FIG. **3** provides a detailed flow chart for an exemplary embodiment that employs reflex sequences for use in sequencing multiple specific regions in a polynucleotide (i.e., regions 1, 2, 3, 4 and 5 in an 11 kb region of lambda DNA).

A single parent DNA fragment 202 is generated that includes adapter domains (i.e., a Roche 454 sequencing primer site, a single MID, and a reflex sequence) and the sequence of interest. In the example shown, the sequence of interest is from lambda DNA and the reflex sequence is present on the top strand (with its complement shown in the bottom strand). Any convenient method for producing this parent DNA fragment may be used, including amplification with a primer that includes the adapter domains (e.g., using PCR), cloning the fragment into a vector that includes the adapter domains (e.g., a vector with the adapter domains adjacent to a cloning site), or by attaching adapters to polynucleotide fragments (e.g., fragment made by random fragmentation, by sequence-specific restriction enzyme digestion, or combinations thereof). While only a single fragment with a single MID is shown, the steps in FIG. **3** are applicable to samples having multiple different fragments each with a different MID, e.g., a sample having a population of homologous fragments from any number of different sources (e.g., different individuals). FIG. **3** describes the subsequent enzymatic steps involved in creating the five daughter fragments in which regions 1, 2, 3, 4 and 5 (shown in polynucleotide 204) are rearranged to be placed within a functional distance of the adapter domains (i.e., close enough to the adapter domains to be sequenced in a single Roche 454 sequencing reaction). Note that certain steps are shown for region 4 only (206).

In step 1, the five regions of interest are defined within the parent fragment (labeled 1 to 5 in polynucleotide 204) and corresponding primer pairs are designed for each. The distance of each region of interest from the reflex sequence is shown below polynucleotide 204. The primer pairs are designed as described and shown in FIG. **1**B (i.e., the A_n-B_n primer pairs). For clarity, only primer sites for region 4 are shown in FIG. **3** ("primer sites" surrounding region 4). In step 2, sequence specific primer extensions are performed (only region 4 is shown) with corresponding B_n primers to produce single stranded polynucleotides having structure 208 (i.e., having the reflex sequence on the 5' terminus). As shown, the B_n primer for region 4 will include a sequence specific primer site that primes at the 3'-most primer site noted for region 4 (where "3'-most" refers to the template strand, which in FIG. **3** is the top strand). This polynucleotide is copied back to produce polynucleotide 210 having reversed polarity (e.g., copied using a primer that hybridizes to the 454A' domain). Polynucleotide 210 has structure similar to polynucleotide 100 shown at the top of FIG. **1**. Step 4 depicts the result of the intramolecular priming between the reflex sequence and its complement followed by extension to produce the MID' and 454A' structures at the 3' end (polynucleotide 212). In the embodiments shown in

FIG. **3**, polynucleotide 212 is treated with T7 exonuclease to remove double stranded DNA from the 5' end (as indicated above, this step is optional). The polynucleotide formed for region 4 is shown as 216 with polynucleotides for the other regions also shown (214).

It is noted here that the formation of each of the polynucleotides 214 may be accomplished either in separate reactions (i.e., structure with region 1 in proximity to the adapter domains is in a first sample, the structure with region 2 in proximity to the adapter region is in a second sample, etc.) or in one or more combined sample.

In step 6 the polynucleotides 214 are copied to reverse polarity to form polynucleotides 218. In step 7, each of these products are then primed with the second primer of the specific primer pair (see A_n primers as shown in FIG. **1**B) each having a second Roche 454 primer site (454B) attached at the 5' end, and extended to form products 220. Steps 6 and 7 may be combined (e.g., in a single PCR or other amplification reaction).

In summary, FIG. **3** shows how the reflex process can be employed to produce five daughter fragments 220 of similar length (e.g., ~500 bp) each of which contain DNA sequences that differ in their distance from the reflex sequence in the starting structure 202 while maintaining the original MID.

FIG. **4** shows another exemplary use of the reflex process as described herein. In the embodiment shown in FIG. **4**, a target sequence (i.e., containing region of interest "E") is enriched from a pool of adapter-attached fragments. In certain embodiments, the fragments are randomly sheared, selected for a certain size range (e.g., DNA having a length from 100 to 5000 base pairs), and tagged with adapters (e.g., asymmetric adapters, e.g., as described in U.S. patent application Ser. No. 12/432,080, filed on Apr. 29, 2009). The asymmetric adaptor employed in FIG. **4** contains a sequencing primer site (454A, as used in the Roche 454 sequencing platform), an MID, an X sequence, and an internal stem region (ISR), which denotes the region of complementarity for the asymmetric adapter that is adjacent to the adapter attachment site (see, e.g., the description in U.S. application Ser. No. 12/432,080, filed on Apr. 29, 2009, incorporated herein by reference in its entirety). The X sequence can be any sequence that can serve as a binding site for a polynucleotide containing the complement of the X sequence (similar to a primer site). As described below, the X sequence allows for the annealing of an oligonucleotide having a 5' overhang that can serve as a template for extension of the 3' end of the adaptor oligonucleotide. The sequencing direction of the sequencing primer site (454A primer site in structure 401 of FIG. **4**) is oriented such that amplification of the adapter ligated fragment using the sequencing primer site proceeds away from the ligated genomic insert. This has the effect of making the initial asymmetric adapter ligated library 'inert' to amplification using this primer, e.g., in a PCR reaction.

To extract a region of interest (the "E" region), the library is mixed with an oligonucleotide (403) containing a 3' X' sequence and a target specific priming sequence (the 1' sequence) under hybridization/annealing conditions. The target specific sequence 1' is designed to flank one side of the region of interest (the 1' sequence adjacent to E in the genomic insert; note that only the E-containing polynucleotide fragment is shown in FIG. **4**), much like a PCR primer. After annealing primer 403, the hybridized complex is extended, whereby all of the adaptor tagged fragments will obtain the complement of the target specific sequence (i.e., the 1 sequence) on the 3' end (see structure 405; arrows denote the direction of extension).

Extended products 405 are then denatured and the 1/1' regions allowed to hybridize intramolecularly in a reflex process priming event, after which nucleic acid extension is performed to form structure 407 (extension is from the 1 priming site; shown with an arrow). This reflex reaction creates a product (407) that, unlike its parent structure (405), has a sequencing primer site (454A) that is oriented such the extension using this primer sequence proceeds towards the region of interest. Thus, in the absence of a priming and extension reflex reaction, extension with a sequencing primer will not generate a product containing the region of interest (the E region). In other words, only E-region containing target polynucleotides will have a 454A sequence that can amplify genomic material (structure 407).

After completing the reflex process (using 1/1' as the reflex sequences), a PCR amplification reaction is performed to amplify the region of interest (with associated adapter domains). However, before performing the PCR reaction, the fragment sample is "inactivated" from further extension using terminal transferase and ddNTPs. This inactivation prevents non-target adaptor tagged molecules from performing primer extension from the 3' primer 1 site. Once inactivated, a PCR reaction is performed using a sequencing primer (i.e., 454A primer 409) and a second primer that primes and extends from the opposite side of the region of interest (i.e., primer 411, which includes a 5' 454B sequencing primer site and a 3' "2" region that primes on the opposite end of E from the 1 region). Only fragments that have undergone the reflex process and contain the E region will be suitable templates for the PCR reaction and produce the desired product (413).

Thus, the process exemplified in FIG. **4** allows for the movement of an adapter domain (e.g., containing functional elements and/or MID) into proximity to a desired region of interest.

The reflex process described herein can be used to perform powerful linkage analysis by combining it with nucleic acid counting methods. Any convenient method for tagging and/or counting individual nucleic acid molecules with unique tags may be employed (see, e.g., U.S. Pat. No. 7,537,897, issued on May 26, 2009; U.S. Pat. No. 7,217,522, issued on May 15, 2007; U.S. patent application Ser. No. 11/377,462, filed on Mar. 16, 2006; and U.S. patent application Ser. No. 12/432,080, filed on Apr. 29, 2009; each of which is incorporated by reference herein in their). All of this can be conducted in parallel thus saving on the cost of labor, time and materials.

In one exemplary embodiment, a large collection of sequences is tagged with MID such that each polynucleotide molecule in the sample has a unique MID. In other words, each polynucleotide in the sample (e.g., each individual double stranded or single stranded polynucleotide) is tagged with a MID that is different from every other MID on every other polynucleotide in the sample. In general, to accomplish such molecular tagging the number of distinct MID tags to be used should be many times greater than the actual number of molecules to be analyzed. This will result in the majority of individual nucleic acid molecules being labeled with a unique ID tag (see, e.g., Brenner et al., Proc. Natl. Acad. Sci. USA. 2000 97(4):1665-70). Any sequences that then result from the reflex process on that particular molecule (e.g., as described above) will thus be labeled with the same unique MID tag and thus inherently linked. Note that once all molecules in a sample are individually tagged, they can be manipulated and amplified as much as needed for processing so long as the MID tag is maintained in the products generated.

For example, we might want to sequence one thousand viral genomes (or a specific genomic region) or one thousand copies of a gene present in somatic cells. After tagging each polynucleotide in the sample with a sequencing primer site, MID and reflex sequence (as shown in the figures and described above), we use the reflex process to break each polynucleotide into lengths appropriate to the sequencing procedure being used, transferring the sequencing primer site and MID to each fragment (as described above). Obtaining sequence information from all of the reflex-processed samples can be used to determine the sequence of each individual polynucleotide in the starting sample, using the MID sequence to defining linkage relationships between sequences from different regions in the polynucleotide being sequenced. Using a sequencing platform with longer read lengths can minimize the number of primers to be used (and reflex fragments generated).

The advantages noted above are shown in FIG. **5**. This figure shows a comparison of methods for identifying nucleic acid polymorphisms in homologous nucleic acids in a sample (e.g., the same region derived from a chromosomal pair of a diploid cell or viral genomes/transcripts). The top schematic shows two nucleic acid molecules in a sample (1 and 2) having a different assortment of polymorphisms in polymorphic sites A, B and C (A1, B1, C1 and C2). Standard sequencing methods using fragmentation (left side) can identify the polymorphisms in these nucleic acids but do not retain linkage information. Employing the reflex process described herein to identify polymorphisms (right side) maintains linkage information. It is noted that not all domain structures and steps are shown in the reflex process for simplicity.

Kits and Systems

Also provided by the subject invention are kits and systems for practicing the subject methods, as described above, such vectors configured to add reflex sequences to nucleic acid inserts of interest and regents for performing any steps in the cloning or reflex process described herein (e.g., restriction enzymes, nucleotides, polymerases, primers, exonucleases, etc.). The various components of the kits may be present in separate containers or certain compatible components may be precombined into a single container, as desired.

The subject systems and kits may also include one or more other reagents for preparing or processing a nucleic acid sample according to the subject methods. The reagents may include one or more matrices, solvents, sample preparation reagents, buffers, desalting reagents, enzymatic reagents, denaturing reagents, where calibration standards such as positive and negative controls may be provided as well. As such, the kits may include one or more containers such as vials or bottles, with each container containing a separate component for carrying out a sample processing or preparing step and/or for carrying out one or more steps of a nucleic acid variant isolation assay according to the present invention.

In addition to above-mentioned components, the subject kits typically further include instructions for using the components of the kit to practice the subject methods, e.g., to prepare nucleic acid samples for perform the reflex process according to aspects of the subject methods. The instructions for practicing the subject methods are generally recorded on a suitable recording medium. For example, the instructions may be printed on a substrate, such as paper or plastic, etc. As such, the instructions may be present in the kits as a package insert, in the labeling of the container of the kit or components thereof (i.e., associated with the packaging or sub-packaging) etc. In other embodiments, the instructions are present as an electronic storage data file present on a suitable computer readable storage medium, e.g. CD-ROM, diskette, etc. In yet other embodiments, the actual instructions are not present in the kit, but means for obtaining the instructions from a remote source, e.g. via the internet, are provided. A_n example of this embodiment is a kit that includes a web address where the instructions can be viewed and/or from which the instructions can be downloaded. As with the instructions, this means for obtaining the instructions is recorded on a suitable substrate.

In addition to the subject database, programming and instructions, the kits may also include one or more control samples and reagents, e.g., two or more control samples for use in testing the kit.

Utility

The reflex process described herein provides significant advantages in numerous applications, a few of which are noted below (as well as described above).

For example, as described above, certain aspects of the reflex process define the particular sections of sequence to be analyzed by a primer pair, as in PCR (e.g., the two oligos shown as A_n-B_n in FIG. **1**B). This results in higher sequence specificity as compared to other extraction methods (e.g., using probes on a microarray) that only use a single oligo sequence. The separation of the probes defines a length that can be relatively uniform (hence making subsequent handling including amplification more uniform) and can also be tailored to the particular sequencing platform being employed.

Further, as described above, aspects of the present invention can be used to analyze homologous genomic locations in a multiplexed sample (i.e., a sample having polynucleotides from different genomic samples) in which the polynucleotides are tagged with the MID. This is possible because the reflex process, which operates intramolecularly, maintains the MID thus linking any particular fragment to the sample from which it originates.

Finally, as the reflex processes described herein function intramolecularly, one can determine the genetic linkage between different regions on the same large fragment that are too far apart to be sequenced in one sequence read. Such a determination of linkage may be of great value in plant or animal genetics (e.g., to decide if a particular set of variations are linked together on the same stretch of chromosome) or in viral studies (e.g., to determine if particular variations are linked together on the same stretch of a viral genome/transcripts, e.g., HIV, hepatitis virus, etc.).

EXAMPLES

Example I

FIGS. **6** and **7** provide experimental data and validation of the reflex process described herein using synthetic polynucleotide substrates.

Methods

Substrate:

The 100 base oligonucleotide substrate (as shown diagrammatically in FIG. **6**A) was synthesized with internal fluorescein-dT positioned between the REFLEX and REFLEX' sequences. This label provides convenient and sensitive method of detection of oligonucleotide species using polyacrylamide gel electrophoresis.

Extension Reactions:

Reactions were prepared which contained 1 μM of the 100 base oligonucleotide substrate, 200 μM dNTPs, presence or

absence of 1 μM competitor oligonucleotide, 0.5 μl of each DNA polymerase ("DNAP"): Vent (NEB, 2 units/μ1), Taq (Qiagen HotStarTaq 5 units/μ1) and Herculase (Stratagene), and made up to 50 μl with the appropriate commercial buffers for each polymerase and dH_2O. For Taq titrations 0.5 μl, 1 μl, 2 μl, and 3 μl enzyme was used in the same 50 μl volume. Reactions were heated in a Biometra thermocycler to 95° C. for 15 minutes (Taq) or 5 minutes (Herculase, Vent), followed by 55° C. or 50° C. for 30 seconds, and a final incubation at 72° C. for 10 minutes.

T7 Exonuclease Digestions:

Reactions were prepared with 10 μl extension reactions above, 0.5 μl T7 exonuclease (NEB, 10 units/μ1), and made up to 50 μl using NEB Buffer 4 and dH_2O. Reactions were incubated at 25° C. for 30 minutes.

Gel Electrophoresis Analysis:

A_n 8% denaturing polyacrylamide gel was used to analyze reaction species. 0.4 μl of extension reactions, and 2 μl of digestion reactions were loaded and ran at 800V for ~1.5 hours. Gels were analyzed for fluorescein using an Amersham/General Electric Typhoon imager.

Results

FIG. **6**A shows the structure of each stage of reflex sequence processing with the expected nucleic acid size shown on the left. The initial single stranded nucleic acid having a sequencing primer site (the Roche 454 sequencing primer A site; listed as 454A); an MID; a reflex sequence; the insert; and a complement of the reflex sequence is 100 nucleotides in length. After self-annealing and extension, the product is expected to be 130 nucleotides in length. After removal of the double stranded region from the 5' end, the nucleic acid is expected to be 82 bases in length.

FIG. **6**B shows the results of three experiments using three different nucleic acid polymerases (Vent, Herculase and Taq, indicated at the top of the lanes). The temperature at which the annealing was carried out is shown at the top of each lane (either 50° C. or 55° C.). The sizes of the three nucleic acids as noted above are indicated on the left and right side of the gel.

As shown in FIG. **6**B, extension appears to be most efficient under the conditions used with Herculase (Herculase is a mixture of two enzymes: modified Pfu DNAP and Archaemax (dUTPase)). Most (or all) of the initial 100 base pair nucleic acid are converted to the 130 base pair product (see lanes 6 and 7). However, after T7 exonuclease digestion the 3'-5' exonuclease activity of Herculase results in partial digestion of the desired 82 base product (note bands at and below the 82 base pairs in lanes 8 and 9).

Taq, which lacks 3'-5' exonuclease activity, shows a stronger band at the expected size of the final product after T7 exonuclease digestion (see lane 13).

FIG. **7** shows the effect on the reflex process of increasing amounts of Taq polymerase as well as the use of a reflex sequence competitor (schematically shown in FIG. **7**A).

As shown in lanes 2 to 5, increased Taq concentration improves extension to ~90% conversion of the starting nucleic acid (see lane 5). Lanes 7 to 8 show that T7 exonuclease digestion does not leave a perfect 82 base product. This may be due to collapse of dsDNA when T7 exonuclease has nearly completed its digestion from the 5' end in the double stranded region of the fold-back structure. It is noted that in many embodiments, the removal of a few additional bases from the 5' end of the polynucleotide will not interfere with subsequent analyses, as nucleotide bases at the 5' end are often removed during subsequent steps.

As shown in Lanes 11-14, addition of a competitor (which can interfere with annealing of the reflex sequences to form the fold-back structure) results in only a small decrease (~5-10%) of fully extended product. Thus, as expected, the intramolecular reaction is heavily favored. Although not shown, we have observed that the competitor oligonucleotide also gets extended by the same amount (~5-10%).

The concentration of the competitor, the concentration of the reflex substrate, and the overall genetic complexity, will all likely affect specific results. The experiments shown in FIGS. **6** and **7** demonstrate that the core parts of the reflex processes as described herein is functional and can be implemented.

Example II

FIG. **8** shows the reflex workflow (diagram at left) and exemplary results of the workflow (gel at right) for a specific region of interest (ROI). The starting material is a double stranded nucleic acid molecule (700) that contains a 454A primer site, an MID, a reflex site, and a polynucleotide of interest having three ROIs (2, 3 and 4) at different locations therein. This starting material was subjected to reflex processes (as described in above) specific for ROI 2 as shown in the diagram at the left of the figure, both with and without the use of a T7 exonuclease step (the T7 exonuclease step is shown in the diagram is indicated as "Optional").

Completion of all steps shown in the reflex process should result in a double stranded polynucleotide of 488 base pairs (702) with or without the T7 exonuclease step.

As shown in the gel on the right of FIG. **8**, the 488 base pair product was produced in reflex processes with and without the T7 exonuclease step.

FIG. **9** shows an exemplary protocol for a reflex process based on the results discussed above. The diagram shows specific reflex process steps with indications on the right as to where purification of reaction products is employed (e.g., using Agencourt SPRI beads to remove primer oligos). One reason for performing such purification steps is to reduce the potential for generating side products in a reaction (e.g., undesirable amplicons). While FIG. **9** indicates three purification steps, fewer or additional purification steps may be employed depending on the desires of the user. It is noted that the steps of reversing polarity, reflex priming and extension, and "stretch out" (or denaturation)/second reversing polarity step can be performed without intervening purification steps.

The protocol shown in FIG. **9** includes the following steps:

- annealing a first primer containing a 5' reflex sequence (or reflex tail, as noted in the figure) specific for the 3' primer site for the R' region to the starting polynucleotide and extending (the primer anneals to the top strand at the primer site at the right of R in polynucleotide 902, indicated with a *; this step represents the first denature, anneal and extend process indicated on the right);
- after purification, adding a 454A primer and performing three cycles of denaturing, annealing and extending: the first cycle results in the copy-back from the 454A primer to reverse the polarity of the strand just synthesized; the second cycle breaks apart the double stranded structure produced, allows the reflex structure to form and then extend; the third cycle results in another copy-back using the same 454A primer originally added;
- after purification, adding a second primer specific for the second primer site for the R' region having a 5' 454B tail (this primer anneals to the primer site 3' of the R'

region in polynucleotide 904, indicated with a *) and denaturing, annealing and extending resulting in a polynucleotide product having 454A and 454B sites surrounding the MID, the reflex sequence, and R'. Note that the first primer specific for the R' region and the second primer specific for the R' region define its boundaries, as described above and depicted in FIG. **1**B);

after another purification, adding 454A and 454B primers and performing a PCR amplification reaction.

Example III

As described above, a reflex sequence can be an "artificial" sequence added to a polynucleotide as part of an adapter or can be based on a sequence present in the polynucleotide of interest being analyzed, e.g., a genomic sequence (or "non-artificial").

The data shown in prior Examples used "artificial" reflex sites. In this Example, the reflex site is a genomic sequence present in the polynucleotide being analyzed.

The starting material is a double stranded DNA containing a 454A site, an MID and a polynucleotide to be analyzed. The 454A and MID were added by adapter ligation to parent polynucleotide fragments followed by enrichment of the polynucleotide to be analyzed by a hybridization-based pull-out reaction and subsequent secondary PCR amplification (see Route 1 in FIG. **13**). Thus, the reflex site employed in this example is a sequence normally present at the 5' end of the subject polynucleotide (a genomic sequence). The polynucleotide being analyzed includes a region of interest distal to the 454A and MID sequences that is 354 base pairs in length.

This starting double stranded nucleic acid is 755 base pairs in length. Based on the length of each of the relevant domains in this starting nucleic acid, the reflex process should result in a product of 461 base pairs.

FIG. **10** shows the starting material for the reflex process (left panel) and the resultant product generated using the reflex process (right panel; reflex process was performed as described in Example II, without using a T7 exonuclease step). A size ladder is included in the left hand lane of each gel to allow estimation of the size of the test material. This figure shows that the 755 base pair starting nucleic acid was processed to the expected 461 base pair product, thus confirming that a "non-artificial" reflex site is effective in moving an adapter domain from one location to another in a polynucleotide of interest in a sequence specific manner.

Example IV

FIG. **11** shows a schematic of an experiment in which the reflex process is performed on a single large initial template (a "parent" fragment) to generate 5 different products ("daughter" products) each having a different region of interest (i.e., daughter products are produced having either region 1, 2, 3, 4 or 5). The schematic in FIG. **11** shows the starting fragment (11,060 base pairs) and resulting products (each 488 base pairs) generated from each of the different region of interest-specific reflex reactions (reflex reactions are performed as described above). The panel (gel) on the bottom of FIG. **11** shows the larger starting fragment (Lane 1) and the resulting daughter products for each region-specific reflex reaction (lanes 2 to 6, with the region of interest noted in each in the box), where the starting and daughter fragments have the expected lengths. Sequencing of the products confirmed the identity of the region of interest in each of the reflex products shown in the gel. These results demonstrate that multiple different reflex products can be generated from a single, asymmetrically tagged parent fragment while maintaining the adapter domains (e.g., the primer sites and MID).

Example V

FIG. **12** details experiments performed to determine the prevalence of intramolecular rearrangement (as desired in the reflex process) vs. intermolecular rearrangement. Intermolecular rearrangement is undesirable because it can lead to the transfer of an MID from one fragment to another (also called MID switching). MID switching can occur if a reflex sequence in a first fragment hybridizes to its complement in a second fragment during the reflex process, leading to appending the MID from the second fragment to the first fragment. Thus, intermolecular rearrangement, or MID switching, should be minimized to prevent the transfer of an MID from one fragment in the sample to another, which could lead to a misrepresentation of the source of a fragment.

To measure the prevalence of MID switching under different reflex conditions, fragments having different sizes were generated that included two different MIDs, as shown in the top panel of FIG. **12**. The common sequence on these fragments serves as the priming site for the first extension reaction to add the second reflex sequence (see, e.g., step 2 of FIG. **3**). Three exemplary fragments are shown in FIG. **12** for each different fragment size (i.e., 800 base pairs with an MIDB and MIDA combination; 1900 base pairs with MIDC and MIDA combination; and 3000 base pairs with MIDD and MIDA combination). For each MID family (A, B, C and D), there are 10 different members (i.e., MIDA had 10 different members, MIDB has 10 different members, etc.). A set of 10 dual MID fragments for each different size fragment (i.e., 800, 1900 and 3000 base pairs) were generated, where the MID pairs (i.e., MIDA/MIDB, MIDA/MIDC, and MIDA/MIDD) were designated as 1/1, 2/2, 3/3, 4/4, 5/5, 6/6, 7/7, 8/8, 9/9, and 10/10. All 10 fragments of the same size were then mixed together and a reflex protocol was performed.

Due to the domain structure of the fragments, a successful reflex process results in the two MIDs for each fragment being moved to within close enough proximity to be sequenced in a single read using the Roche 454 sequencing platform (see the reflex products shown in the schematic of FIG. **12**). The reflex reactions for each fragment size were performed at four different fragment concentrations to determine the effect of this parameter, as well as fragment length, in the prevalence of MID switching. The reflex products from each reaction performed were subjected to 454 sequencing to determine the identity of both MIDs on each fragment, and thereby the proportion of MID switching that occurred.

The panel on the bottom left of FIG. **12** shows the rate of MID switching (Y axis, shown in % incorrect (or switched) MID pair) for each different length fragment at each different concentration (X axis; 300, 30, 3 and 0.3 nM). As shown in this panel, the MID switch rate decreases with lower concentrations, as would be expected, because intermolecular, as opposed to intramolecular, binding events are concentration dependent (i.e., lower concentrations lead to reduced intermolecular hybridization/binding). In addition, the MID switch rate decreases slightly with length. This is somewhat unexpected as the ends of longer DNA fragments are effectively at a lower concentration with respect to one

another. The reasons for why we do not see this is probably because the production of reflex priming intermediates continues during the final PCR, which means that reflex priming reactions are happening continuously which contributes to MID switching. It is probably the case that the shorter reflex products are able to undergo a higher rate of 'background' reflexing, and therefore increase the overall MID switch rate a little.

These results demonstrate that MID switching can be minimized (e.g., to below 2%, below 1% or even to nearly undetectable levels) by altering certain parameters of the reaction, e.g., by reducing fragment concentration and/or fragment length.

The panel on the bottom right of FIG. **12** shows the frequency of MID switching in the reflex process for the 800 base pair fragments (i.e., MIDA/MIDB containing fragments). In this figure, the area of each circle is proportional to the number of reads containing the corresponding MIDA and MIDB species (e.g., MIDA1/MIDB1; MIDA1/MIDB2; etc.). Thus, a circle representing 200 reads will be 40 times larger in terms of area than a circle representing 5 reads.

As noted above, the MIDA/MIDB combinations having the same number (shown on the X and Y axis, respectively) represent the MIDA/MIDB combinations present in the sample prior to the reflex process being performed (i.e., MIDA/MIDB combinations 1/1, 2/2, 3/3, 4/4, 5/5, 6/6, 7/7, 8/8, 9/9, and 10/10 were present in the starting sample). All other MIDA/MIDB combinations identified by Roche 454 sequencing were the result of MID switching.

This figure shows that the MID switching that occurs during the reflex process is random, i.e., that MID switching is not skewed based on the identity of the MIDB in the reaction).

Exemplary Reflex Protocols

FIG. **13** shows a diagram of exemplary protocols for performing the reflex process on pools of nucleic acids, for example, pools of nucleic acids from different individuals, each of which are labeled with a unique MID. In Route 3, a pooled and tagged extended library is subjected directly to a reflex process. In Route 2, the pooled library is enriched by target-specific hybridization followed by performing the reflex process. In Route 1 employs enrichment by PCR amplification. As shown in FIG. **13**, PCR enrichment can be performed directly on the pooled tagged extended library or in a secondary PCR reaction after a hybridization-based enrichment step has been performed (as in Route 2) to generate an amplicon substrate that is suitable for the reflex process. Additional routes for preparing a polynucleotide sample for performing a reflex process can be implemented (e.g., having additional amplification, purification, and/or enrichment steps), which will generally be dependent on the desires of the user.

Although the foregoing invention has been described in some detail by way of illustration and example for purposes of clarity of understanding, it is readily apparent to those of ordinary skill in the art in light of the teachings of this invention that certain changes and modifications may be made thereto without departing from the spirit or scope of the appended claims.

Accordingly, the preceding merely illustrates the principles of the invention. It will be appreciated that those skilled in the art will be able to devise various arrangements which, although not explicitly described or shown herein, embody the principles of the invention and are included within its spirit and scope. Furthermore, all examples and conditional language recited herein are principally intended to aid the reader in understanding the principles of the invention and the concepts contributed by the inventors to furthering the art, and are to be construed as being without limitation to such specifically recited examples and conditions. Moreover, all statements herein reciting principles, aspects, and embodiments of the invention as well as specific examples thereof, are intended to encompass both structural and functional equivalents thereof. Additionally, it is intended that such equivalents include both currently known equivalents and equivalents developed in the future, i.e., any elements developed that perform the same function, regardless of structure. The scope of the present invention, therefore, is not intended to be limited to the exemplary embodiments shown and described herein. Rather, the scope and spirit of present invention is embodied by the appended claims.

What is claimed:

1. A method for multiplexed analysis of nucleic acids from single cells, the method comprising:

(a) providing a sample comprising a plurality of cells, wherein a single cell of the plurality of cells comprises a plurality of sample polynucleotides;

(b) performing primer extension to generate a plurality of tagged polynucleotides from said plurality of sample polynucleotides and a plurality of oligonucleotide tags, wherein a tagged polynucleotide of the plurality of tagged polynucleotides comprises:

(i) a sample sequence from a sample polynucleotide of the plurality of sample polynucleotides;

(ii) a first tag sequence distinguishing said sample polynucleotide from sample polynucleotides originating from other single cells; and

(iii) a second tag sequence distinguishing said sample polynucleotide from other sample polynucleotides originating from said same single cell;

(c) amplifying said tagged polynucleotide, thereby generating a plurality of amplified polynucleotides; and

(d) sequencing said plurality of amplified polynucleotides to determine sequences of the amplified polynucleotides corresponding to the sample sequence, the first tag sequence, and the second tag sequence of the tagged polynucleotide; and

(e) using the sequences determined in step (d) to count sample polynucleotides for multiple different sample polynucleotides of multiple different single cells of said plurality of cells.

2. The method of claim **1**, wherein the plurality of sample polynucleotides comprise messenger RNA (mRNA).

3. The method of claim **1**, wherein an oligonucleotides tag of said plurality of oligonucleotide tags comprises said first tag sequence and said second tag sequence.

4. The method of claim **1**, wherein an oligonucleotides tag of said plurality of oligonucleotide tags comprises a sequence that is configured to hybridize to said sample polynucleotides.

5. The method of claim **1**, wherein step (b) comprises (i) hybridizing an oligonucleotide tag of said plurality of oligonucleotide tags to said sample polynucleotide and (ii) extending said oligonucleotide tag or said sample polynucleotide or both.

6. The method of claim **2**, wherein step (b) comprises (i) hybridizing an oligonucleotide tag of said plurality of oligonucleotide tags to said mRNA and (ii) extending said oligonucleotide tag using said mRNA as a template to generate complementary DNA (cDNA).

7. The method of claim **6**, wherein said hybridizing comprises hybridizing a poly-dT sequence of said oligonucleotide tag to a poly-dA sequence of said mRNA.

8. The method of claim 1, wherein step (b) comprises randomly associating said plurality of sample polynucleotides with said plurality of oligonucleotide tags.

9. The method of claim 1, wherein said plurality of oligonucleotide tags comprise second tag sequences that are random sequences.

10. The method of claim 1, wherein step (c) comprises generating said plurality of amplified polynucleotides using polymerase chain reaction (PCR).

11. The method of claim 1, wherein substantially every sample polynucleotide of said plurality of sample polynucleotides is associated with the same first tag sequence.

12. The method of claim 1, wherein at least 90 percent of said plurality of tagged polynucleotides have a second tag sequence that is different from second tag sequences of the other tagged polynucleotides.

13. The method of claim 1, wherein substantially every one of said plurality of tagged polynucleotides has a second tag sequence that is different from second tag sequences of the other tagged polynucleotides.

14. The method of claim 1, wherein said plurality of oligonucleotide tags comprises a number of different second tag sequences that is larger than the number of sample polynucleotides.

15. The method of claim 14, wherein said plurality of oligonucleotide tags comprises a number of different second tag sequences that is at least ten times the number of sample polynucleotides.

16. The method of claim 15, wherein said plurality of oligonucleotide tags comprises a number of different second tag sequences that is at least one hundred times the number of sample polynucleotides.

17. The method of claim 1, wherein said plurality of oligonucleotide tags comprises at least 200,000 different second tag sequences.

18. The method of claim 1, wherein an oligonucleotides tag of said plurality of oligonucleotide tags comprises a sequencing adaptor.

19. The method of claim 18, wherein step (d) comprises hybridizing said amplified polynucleotides or derivatives thereof to a solid support via said sequencing adaptor or derivative thereof.

20. The method of claim 19, wherein said solid support is a bead.

21. The method of claim 1, wherein step (e) comprises using second tag sequences of said plurality of amplified polynucleotides to determine that said plurality of amplified polynucleotides are amplified from said tagged polynucleotide.

22. The method of claim 1, wherein step (e) comprises using sample sequences, first tag sequences, and second tag sequences of said plurality of amplified polynucleotides to determine that said plurality of amplified polynucleotides are amplified from said tagged polynucleotide.

23. The method of claim 1, wherein step (e) comprises determining the number of different second tags sequences associated with said sample sequence, thereby estimating the number of sample polynucleotides having said sample sequence from said single cell.

24. The method of claim 1, wherein step (e) comprises using second tag sequences of said plurality of amplified polynucleotides to provide a digital count of said sample polynucleotides.

25. The method of claim 1, wherein said plurality of oligonucleotide tags are generated by combinatorial synthesis from a defined set of subunits.

26. The method of claim 1, wherein said plurality of sample polynucleotides are pooled with sample polynucleotides from other single cells of said plurality of cells prior to the generating of step (b).

27. The method of claim 1, wherein said plurality of tagged polynucleotides are pooled with tagged polynucleotides from other single cells of said plurality of cells prior to the amplifying of step (c).

28. The method of claim 1, wherein said plurality of amplified polynucleotides are pooled with amplified polynucleotides from other single cells of said plurality of cells prior to the sequencing of step (d).

29. The method of claim 1, wherein step (e) comprises using first tag sequences of said plurality of amplified polynucleotides to correlate the sample sequences of said plurality of amplified polynucleotides with the single cell from which the sample sequences are derived based on amplified polynucleotides from the same cell having the same first tag sequence.

30. The method of claim 1, wherein step (e) comprises using second tag sequences of said plurality of amplified polynucleotides to correlate the sample sequences of said plurality of amplified polynucleotides with the sample polynucleotide from which the sample sequences are derived based on amplified polynucleotides from the same sample polynucleotide having the same second tag sequence.

* * * * *

Exhibit E

US010392662B1

(12) **United States Patent**
Brenner et al.

(10) **Patent No.: US 10,392,662 B1**
(45) **Date of Patent: *Aug. 27, 2019**

(54) **METHODS FOR ANALYZING NUCLEIC ACIDS FROM SINGLE CELLS**

(71) Applicant: **10X Genomics, Inc.**, Pleasanton, CA (US)

(72) Inventors: **Sydney Brenner**, Ely (GB); **Gi Mikawa**, Great Shelford (GB); **Robert Osborne**, Great Chesterford (GB); **Andrew Slatter**, London (GB)

(73) Assignee: **10X GENOMICS, INC.**, Pleasanton, CA (US)

(*) Notice: Subject to any disclaimer, the term of this patent is extended or adjusted under 35 U.S.C. 154(b) by 0 days.

This patent is subject to a terminal disclaimer.

(21) Appl. No.: **16/397,832**

(22) Filed: **Apr. 29, 2019**

Related U.S. Application Data

(63) Continuation of application No. 16/282,188, filed on Feb. 21, 2019, now Pat. No. 10,337,063, which is a continuation of application No. 16/261,268, filed on Jan. 29, 2019, now Pat. No. 10,280,459, which is a continuation of application No. 16/194,047, filed on Nov. 16, 2018, now Pat. No. 10,240,197, which is a continuation of application No. 15/677,957, filed on Aug. 15, 2017, now Pat. No. 10,155,981, which is a continuation of application No. 14/792,094, filed on Jul. 6, 2015, now abandoned, which is a continuation of application No. 14/172,694, filed on Feb. 4, 2014, now Pat. No. 9,102,980, which is a continuation of application No. 14/021,790, filed on Sep. 9, 2013, now Pat. No. 8,679,756, which is a continuation of application No. 13/859,450, filed on Apr. 9, 2013, now Pat. No. 8,563,274, which is a continuation of application No. 13/622,872, filed on Sep. 19, 2012, now abandoned, which is a continuation of application No. 13/387,343, filed as application No. PCT/IB2010/002243 on Aug. 13, 2010, now Pat. No. 8,298,767.

(60) Provisional application No. 61/288,792, filed on Dec. 21, 2009, provisional application No. 61/235,595, filed on Aug. 20, 2009.

(51) **Int. Cl.**

C12Q 1/6874	(2018.01)
C12Q 1/6855	(2018.01)
C12Q 1/6806	(2018.01)
C12N 15/10	(2006.01)
C12Q 1/686	(2018.01)

(52) **U.S. Cl.**
CPC ***C12Q 1/6874*** (2013.01); ***C12N 15/1065*** (2013.01); ***C12Q 1/6806*** (2013.01); ***C12Q 1/686*** (2013.01); ***C12Q 1/6855*** (2013.01)

(58) **Field of Classification Search**
CPC C12Q 1/6874; C12N 15/1065
USPC 506/9
See application file for complete search history.

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Primary Examiner — Karla A Dines
(74) *Attorney, Agent, or Firm* — Morgan, Lewis & Bockius LLP

(57) **ABSTRACT**

Aspects of the present invention include analyzing nucleic acids from single cells using methods that include using tagged polynucleotides containing multiplex identifier sequences.

13 Claims, 13 Drawing Sheets

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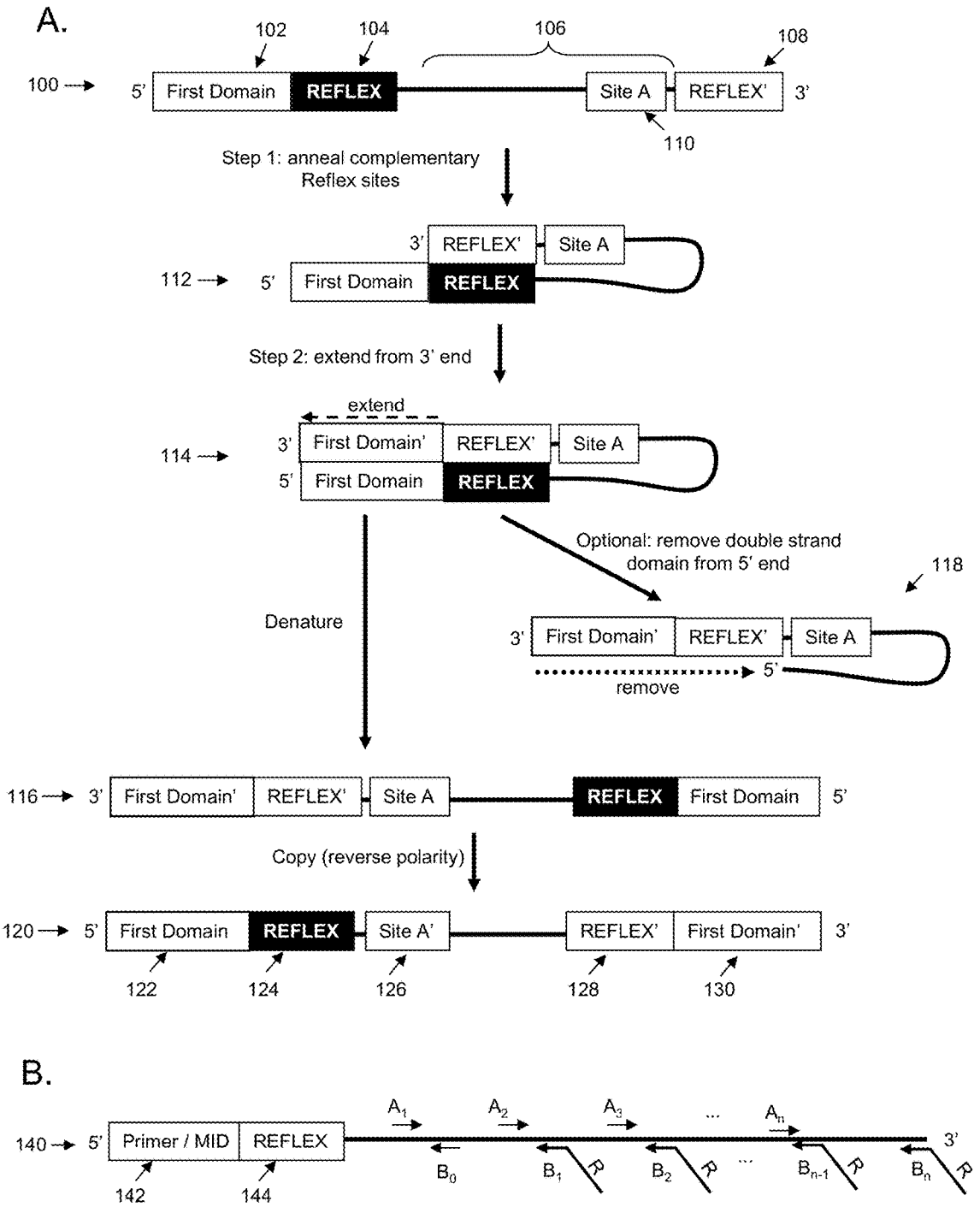

A.
100
5'
First Domain
REFLEX
102
104
106
108
Site A
REFLEX'
3'
110
Step 1: anneal complementary Reflex sites
112
3'
REFLEX'
Site A
5'
First Domain
REFLEX
Step 2: extend from 3' end
extend
114
3'
First Domain'
REFLEX'
Site A
5'
First Domain
REFLEX
Denature
Optional: remove double strand domain from 5' end
118
3'
First Domain'
REFLEX'
Site A
5'
remove
116
3'
First Domain'
REFLEX'
Site A
REFLEX
First Domain
5'
Copy (reverse polarity)
120
5'
First Domain
REFLEX
Site A'
REFLEX'
First Domain'
3'
122
124
126
128
130
B.
140
5'
Primer / MID
REFLEX
142
144
A1
A2
A3
...
An
3'
B0
B1
P
B2
P
...
Bn-1
P
Bn
P

Fig. 1

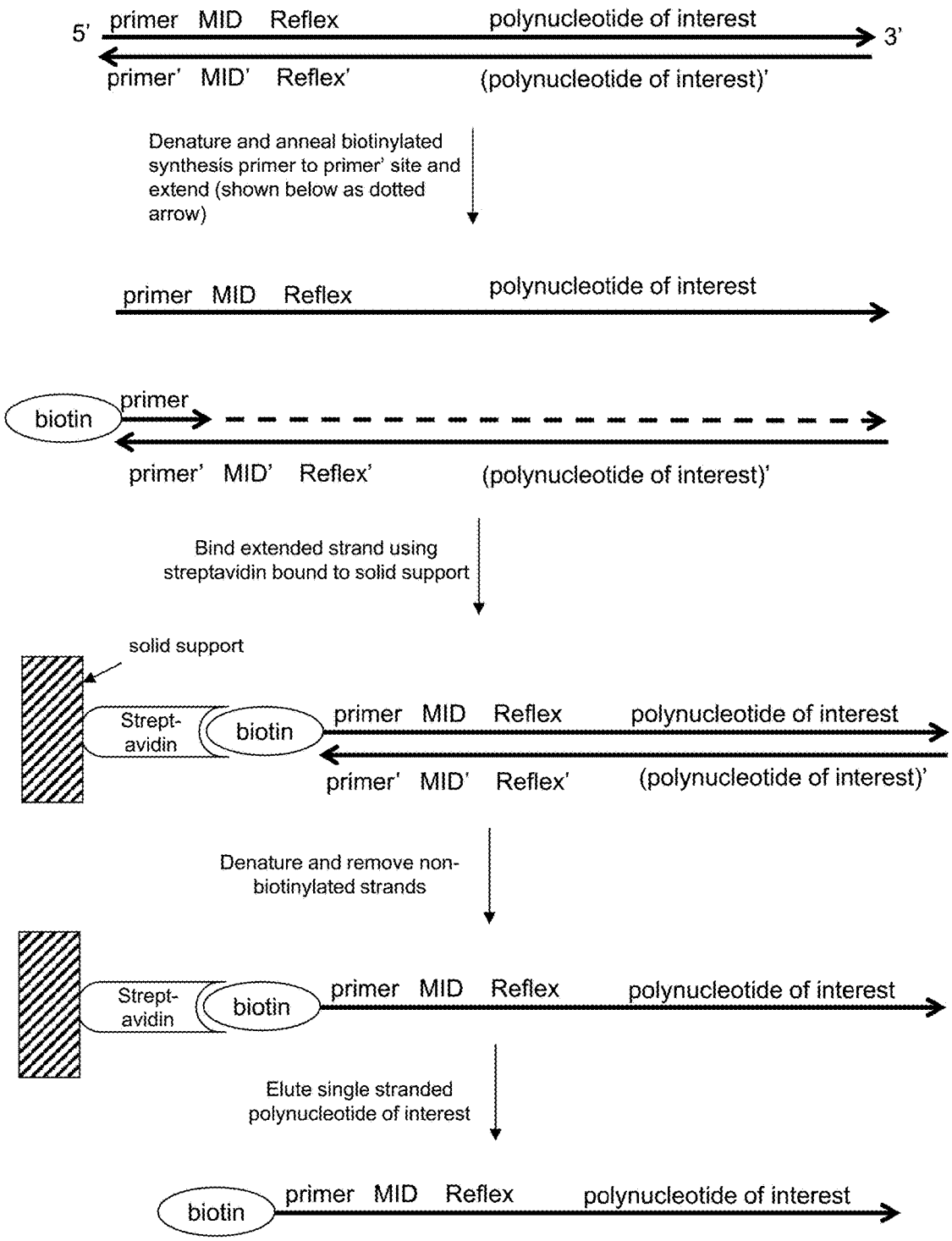
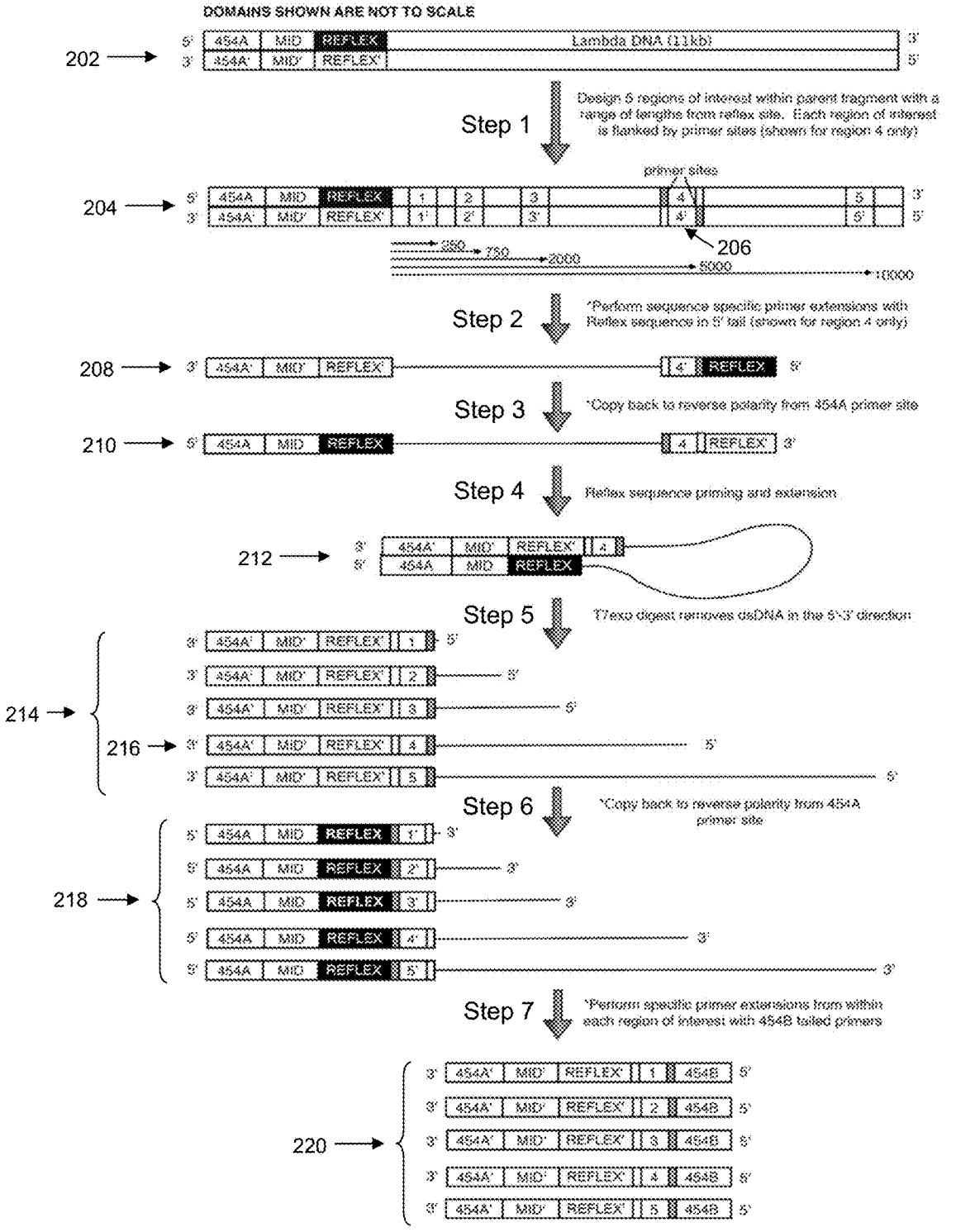

5'
primer MID Reflex
polynucleotide of interest
3'
primer' MID' Reflex'
(polynucleotide of interest)'
Denature and anneal biotinylated synthesis primer to primer' site and extend (shown below as dotted arrow)
primer MID Reflex
polynucleotide of interest
biotin
primer
primer' MID' Reflex'
(polynucleotide of interest)'
Bind extended strand using streptavidin bound to solid support
solid support
Strept-avidin
biotin
primer MID Reflex
polynucleotide of interest
primer' MID' Reflex'
(polynucleotide of interest)'
Denature and remove non-biotinylated strands
Strept-avidin
biotin
primer MID Reflex
polynucleotide of interest
Elute single stranded polynucleotide of interest
biotin
primer MID Reflex
polynucleotide of interest

Fig. 2



Primer extension reactions with * may be performed such that isolation of single strand species is facilitated (e.g., using primers with binding moieties and/or multiple cycles of extension)

Fig. 3

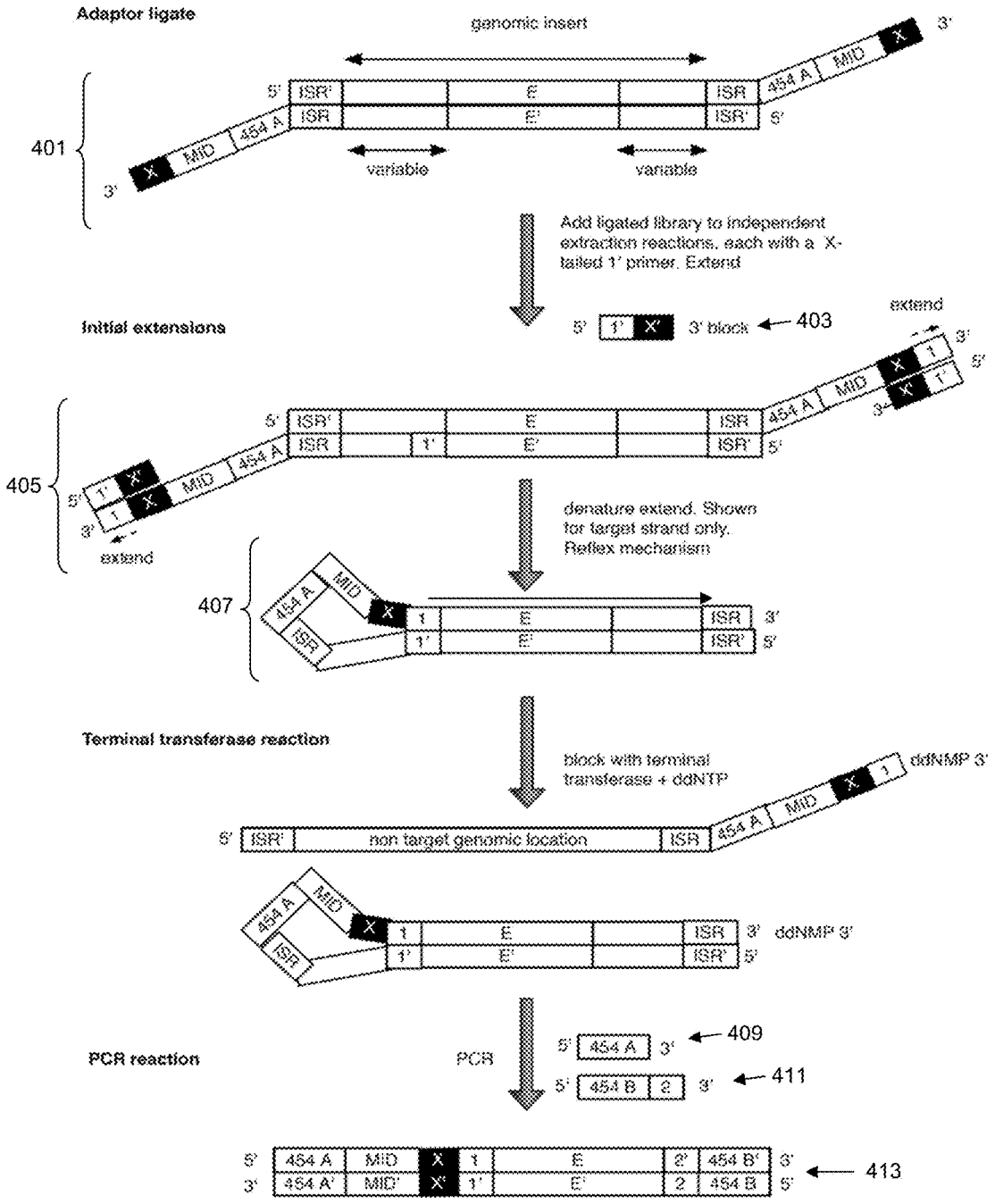

Adaptor ligate
genomic insert
401
variable
variable
Add ligated library to independent extraction reactions, each with a X-tailed 1' primer. Extend
Initial extensions
403
extend
405
extend
denature extend. Shown for target strand only. Reflex mechanism
407
Terminal transferase reaction
block with terminal transferase + ddNTP
ddNMP 3'
non target genomic location
PCR reaction
PCR
409
411
413

Fig. 4

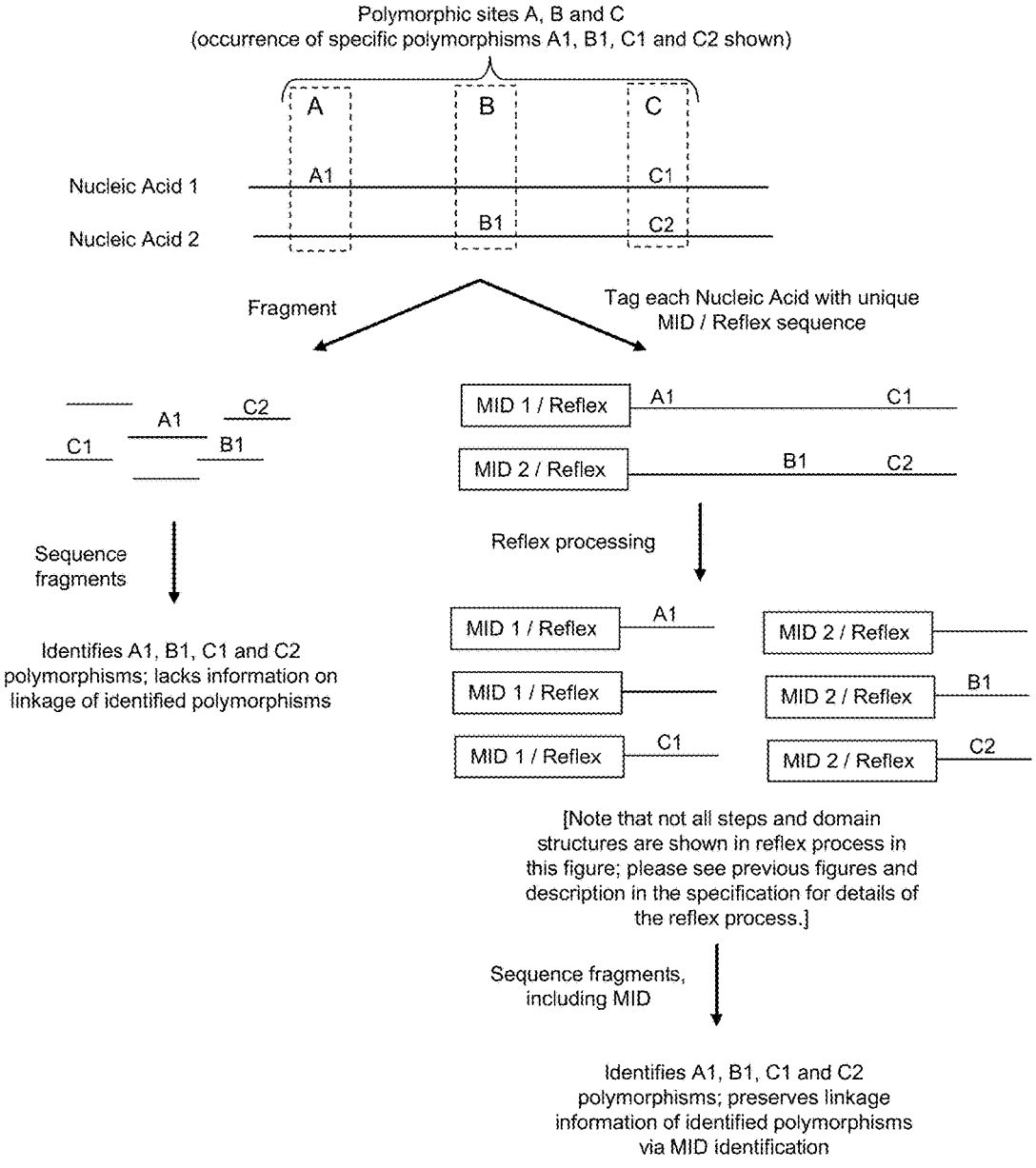
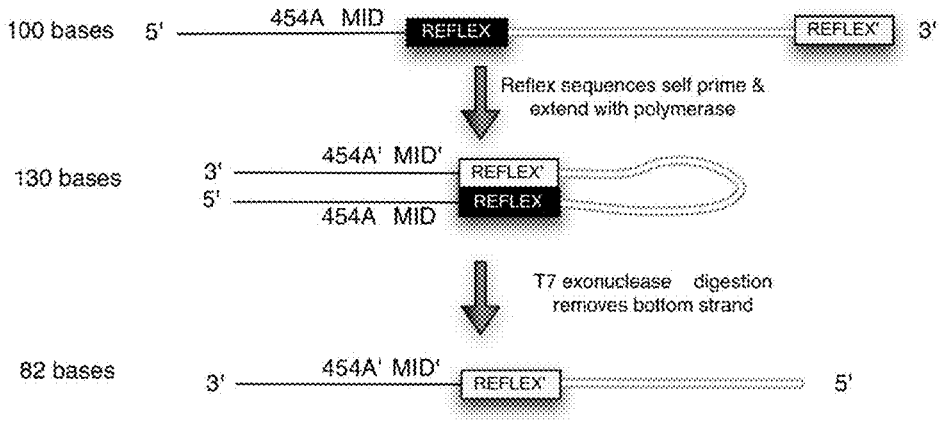
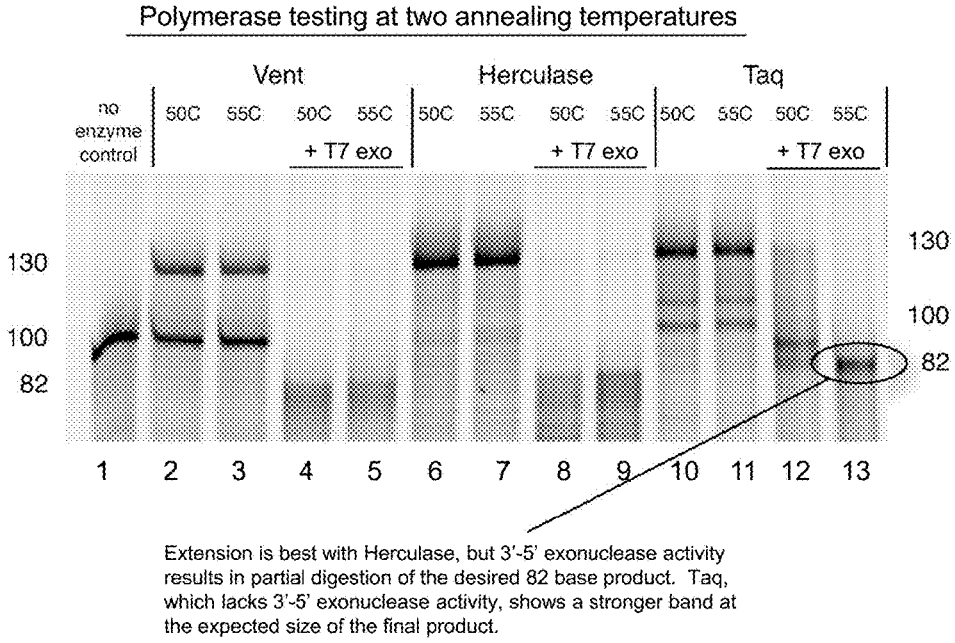

Polymorphic sites A, B and C
(occurrence of specific polymorphisms A1, B1, C1 and C2 shown)
A
B
C
Nucleic Acid 1
A1
C1
Nucleic Acid 2
B1
C2
Fragment
Tag each Nucleic Acid with unique MID / Reflex sequence
A1
C2
C1
B1
MID 1 / Reflex
A1
C1
MID 2 / Reflex
B1
C2
Sequence fragments
Reflex processing
Identifies A1, B1, C1 and C2 polymorphisms; lacks information on linkage of identified polymorphisms
MID 1 / Reflex
A1
MID 2 / Reflex
MID 1 / Reflex
MID 2 / Reflex
B1
MID 1 / Reflex
C1
MID 2 / Reflex
C2
[Note that not all steps and domain structures are shown in reflex process in this figure; please see previous figures and description in the specification for details of the reflex process.]
Sequence fragments, including MID
Identifies A1, B1, C1 and C2 polymorphisms; preserves linkage information of identified polymorphisms via MID identification

Fig. 5

A.



B.



Extension is best with Herculase, but 3'-5' exonuclease activity results in partial digestion of the desired 82 base product. Taq, which lacks 3'-5' exonuclease activity, shows a stronger band at the expected size of the final product.

Fig. 6

A.


5'
454A MID
REFLEX
REFLEX'
3'
+ Competitor
5'
REFLEX'
3'

B.

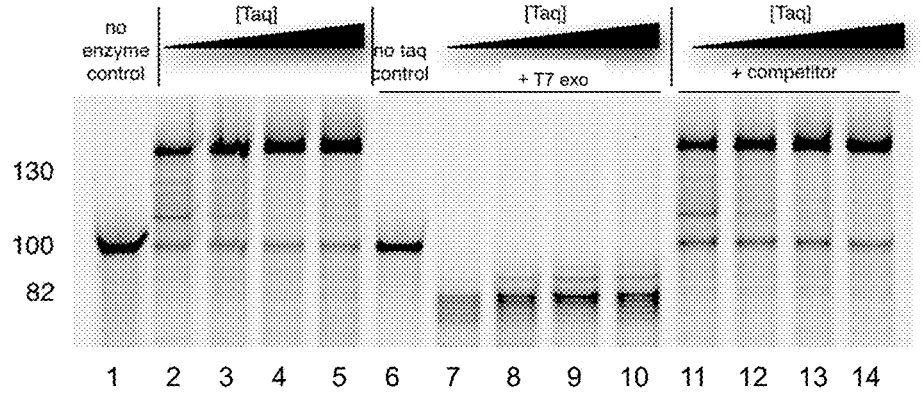
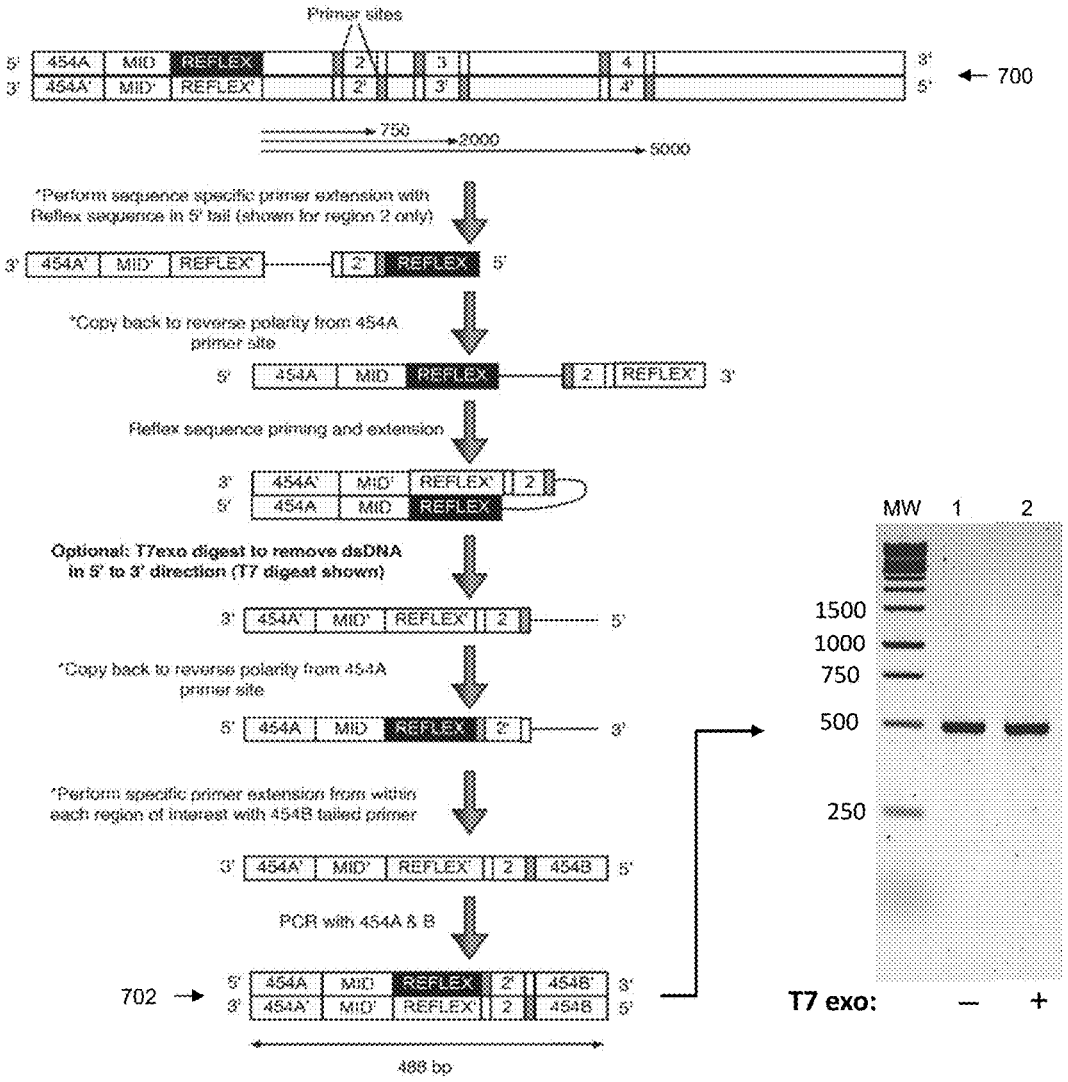

no enzyme control
[Taq]
no taq control
[Taq]
+ T7 exo
[Taq]
+ competitor
130
100
82
1 2 3 4 5 6 7 8 9 10 11 12 13 14

Fig. 7



Primer extension reactions with * may be performed such that isolation of single strand species is facilitated (e.g., using primers with binding moieties and/or multiple cycles of extension)

Fig. 8

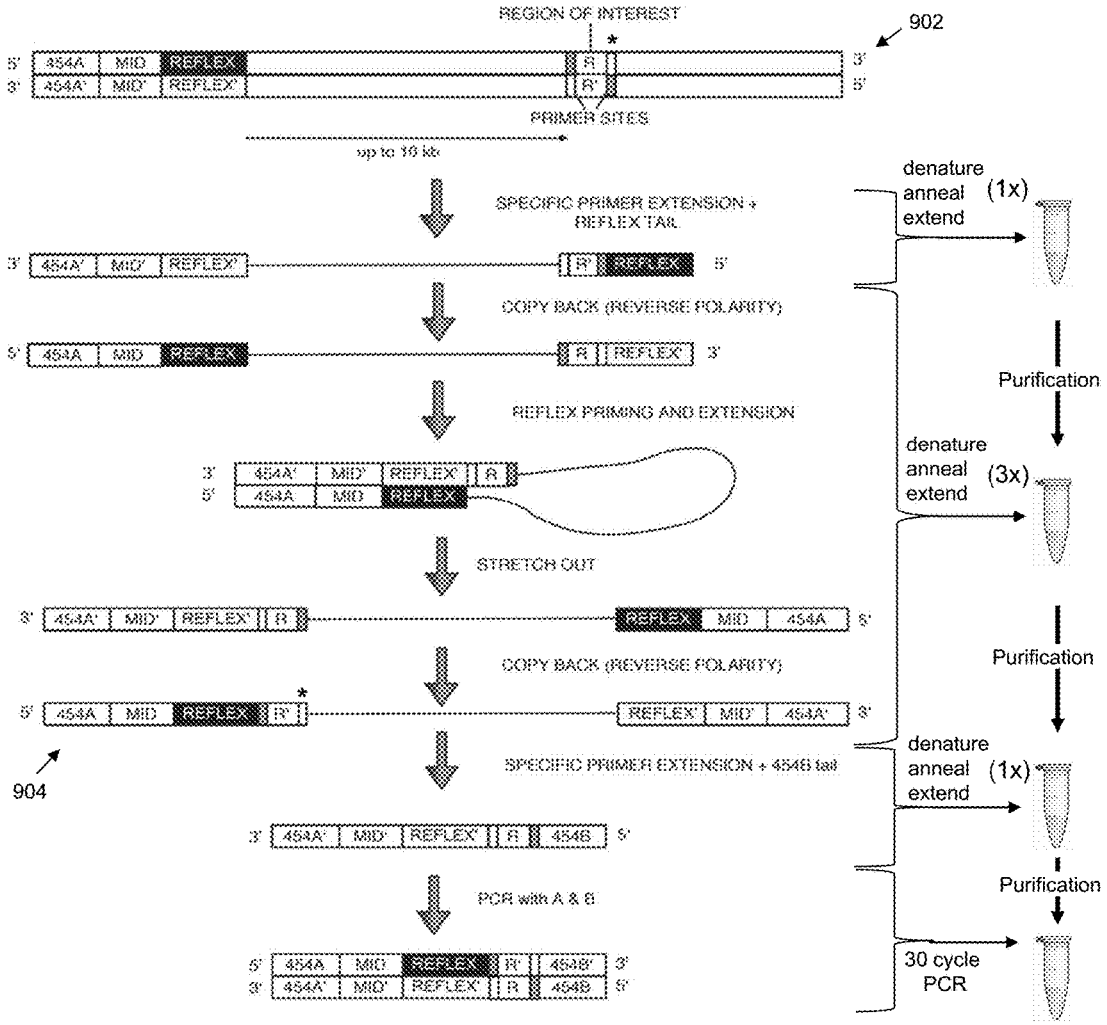

REGION OF INTEREST
902
PRIMER SITES
up to 10 kb
SPECIFIC PRIMER EXTENSION + REFLEX TAIL
COPY BACK (REVERSE POLARITY)
REFLEX PRIMING AND EXTENSION
STRETCH OUT
COPY BACK (REVERSE POLARITY)
SPECIFIC PRIMER EXTENSION + 454B tail
PCR with A & B
904
denature anneal extend (1x)
Purification
denature anneal extend (3x)
Purification
denature anneal extend (1x)
Purification
30 cycle PCR

Fig. 9

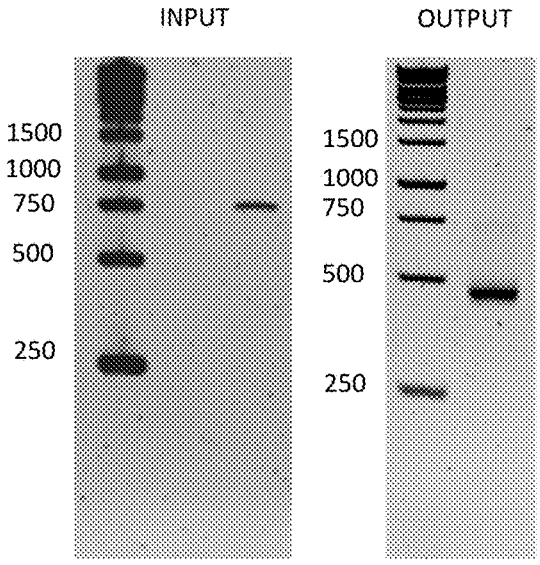
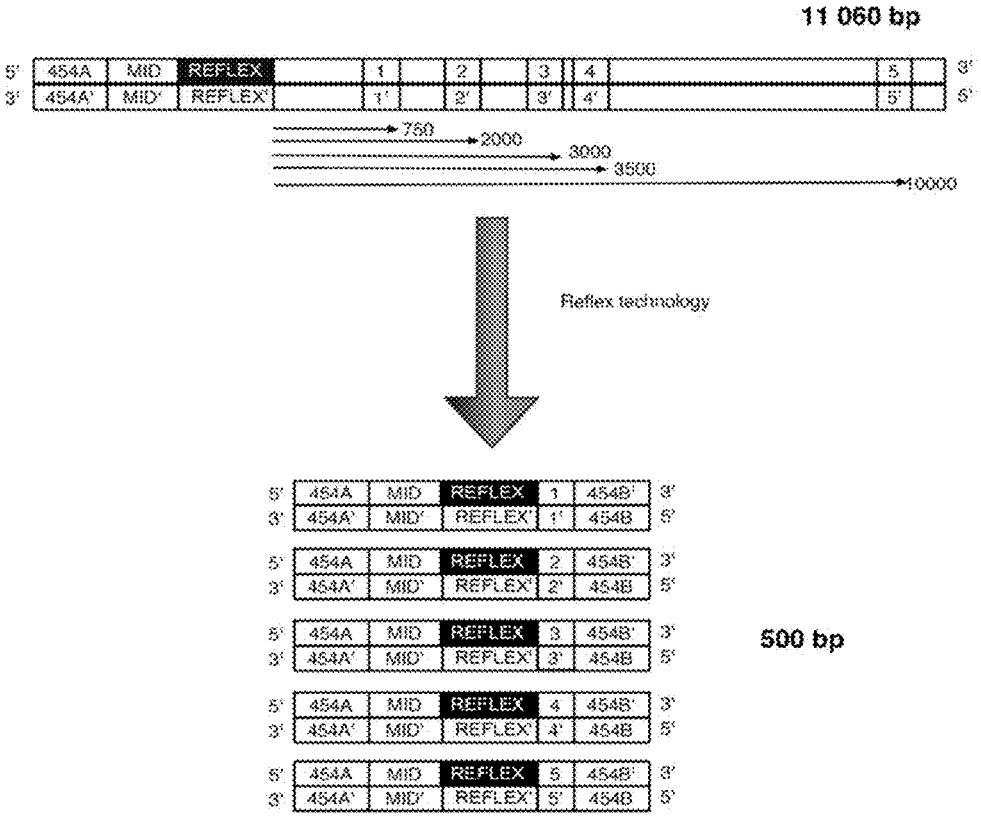
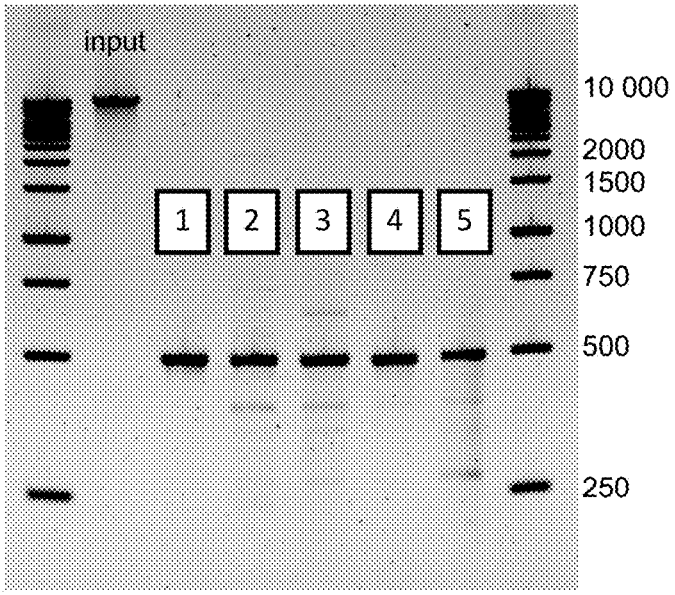


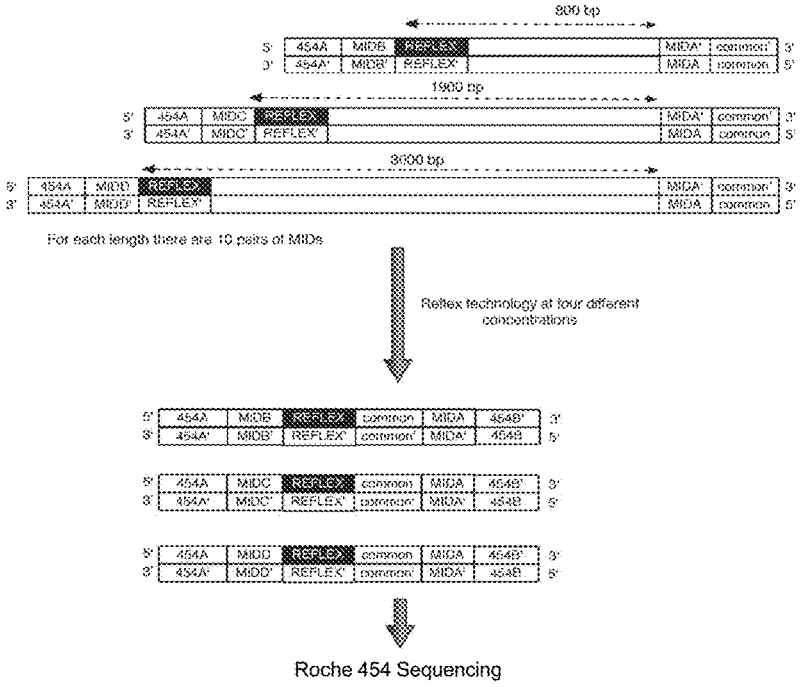
Fig. 10

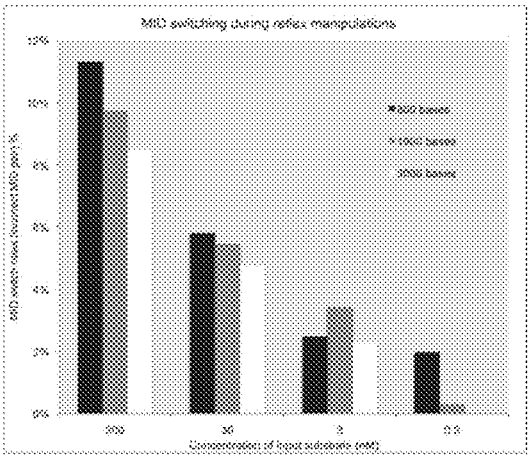




Lanes: 1 2 3 4 5 6

Fig. 11


800 bp
1900 bp
3600 bp
454A
MID
REFLEX
MIDA
common
454B
For each length there are 10 pairs of MIDs
Reflex technology at four different concentrations
Roche 454 Sequencing


MID switching during reflex manipulations
800 bases
1900 bases
3600 bases

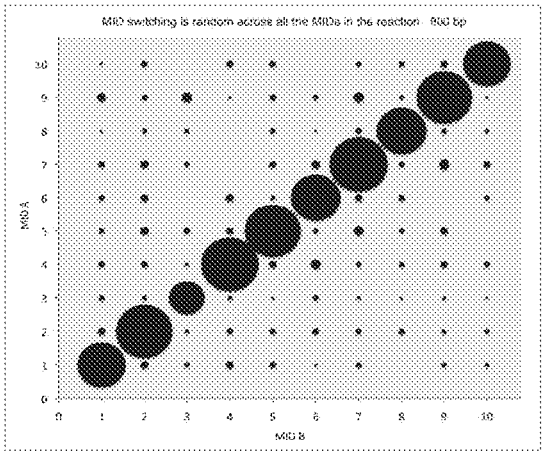

MID switching is random across all the MIDs in the reaction - 800 bp
MID A
MID B

Fig. 12

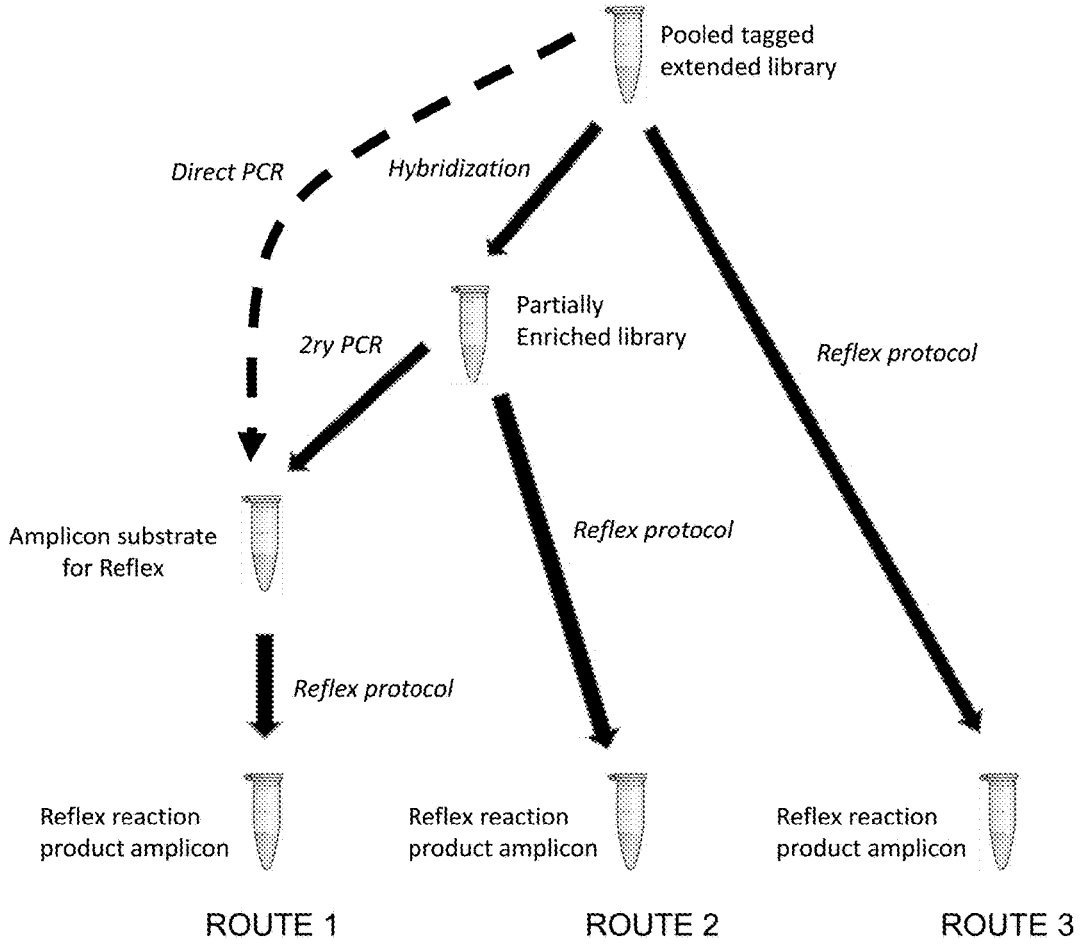

Pooled tagged extended library
Direct PCR
Hybridization
Partially Enriched library
2ry PCR
Reflex protocol
Amplicon substrate for Reflex
Reflex protocol
Reflex protocol
Reflex reaction product amplicon
Reflex reaction product amplicon
Reflex reaction product amplicon
ROUTE 1
ROUTE 2
ROUTE 3

Fig. 13

METHODS FOR ANALYZING NUCLEIC ACIDS FROM SINGLE CELLS

CROSS REFERENCE TO RELATED APPLICATIONS

This application is a continuation of U.S. application Ser. No. 16/282,188, filed Feb. 21, 2019, which is a continuation of U.S. application Ser. No. 16/261,268, filed Jan. 29, 2019, now U.S. Pat. No. 10,280,459, which is a continuation of U.S. application Ser. No. 16/194,047, filed Nov. 16, 2018, now U.S. Pat. No. 10,240,197, which is a continuation of U.S. application Ser. No. 15/677,957, filed Aug. 15, 2017, now U.S. Pat. No. 10,155,981, which is a continuation of U.S. application Ser. No. 14/792,094, filed Jul. 6, 2015, which is a continuation of U.S. application Ser. No. 14/172,694, filed Feb. 4, 2014, now U.S. Pat. No. 9,102,980, which is a continuation of U.S. application Ser. No. 14/021,790, filed Sep. 9, 2013, now U.S. Pat. No. 8,679,756, which is a continuation of U.S. application Ser. No. 13/859,450, filed Apr. 9, 2013, now U.S. Pat. No. 8,563,274, which is a continuation of U.S. application Ser. No. 13/622,872, filed Sep. 19, 2012, which is a continuation of U.S. application Ser. No. 13/387,343, filed Feb. 15, 2012, now U.S. Pat. No. 8,298,767, which is a § 371 National Phase Application of PCT/IB2010/002243, filed Aug. 13, 2010, which claims priority to U.S. Provisional Application No. 61/235,595, filed Aug. 20, 2009 and U.S. Provisional Application No. 61/288,792, filed Dec. 21, 2009; all of which are herein incorporated by reference.

BACKGROUND OF THE INVENTION

We have previously described methods that enable tagging each of a population of fragmented genomes and then combining them together to create a 'population library' that can be processed and eventually sequenced as a mixture. The population tags enable analysis software to parse the sequence reads into files that can be attributed to a particular genome in the population. One limitation of the overall process stems from limitations of existing DNA sequencing technologies. In particular, if fragments in the regions of interest of the genome are longer than the lengths that can be sequenced by a particular technology, then such fragments will not be fully analyzed (since sequencing proceeds from an end of a fragment inward). Furthermore, a disadvantage of any sequencing technology dependent on fragmentation is that sequence changes in one part of a particular genomic region may not be able to be linked to sequence changes in other parts of the same genome (e.g., the same chromosome) because the sequence changes reside on different fragments. (See FIG. **5** and its description below).

The present invention removes the limitations imposed by current sequencing technologies as well as being useful in a number of other nucleic acid analyses.

SUMMARY OF THE INVENTION

Aspects of the present invention are drawn to processes for moving a region of interest in a polynucleotide from a first position to a second position with regard to a domain within the polynucleotide, also referred to as a "reflex method" (or reflex process, reflex sequence process, reflex reaction, and the like). In certain embodiments, the reflex method results in moving a region of interest into functional proximity to specific domain elements present in the polynucleotide (e.g., primer sites and/or MID). Compositions, kits and systems that find use in carrying out the reflex processes described herein are also provided.

BRIEF DESCRIPTION OF THE DRAWINGS

The invention is best understood from the following detailed description when read in conjunction with the accompanying drawings. It is emphasized that, according to common practice, the various features of the drawings are not to scale. Indeed, the dimensions of the various features are arbitrarily expanded or reduced for clarity. Included in the drawings are the following figures:

FIG. **1**: Panel A is a schematic diagram illustrating moving a first domain from one site to another in a nucleic acid molecule using a reflex sequence. Panel B is a schematic diagram depicting the relative position of primer pairs (A_n-B_n primers) that find use in aspects of the reflex process described herein.

FIG. **2** shows an exemplary embodiment of using binding partner pairs (biotin/streptavidin) to isolate single stranded polynucleotides of interest.

FIG. **3** is a schematic diagram illustrating an exemplary embodiment for moving a primer site and a MID to a specific location in a nucleic acid of interest.

FIG. **4** shows a schematic diagram illustrating an exemplary use of the reflex process for generating a sample enriched for fragments having a region of interest (e.g., from a population of randomly fragmented and asymmetrically tagged polynucleotides).

FIG. **5** shows a comparison of methods for identifying nucleic acid polymorphisms in homologous nucleic acids in a sample (e.g., the same region derived from a chromosomal pair of a diploid cell or viral genomes/transcripts). The top schematic shows two nucleic acid molecules in a sample (1 and 2) having a different assortment of polymorphisms in polymorphic sites A, B and C (A1, B1, C1 and C2). Standard sequencing methods using fragmentation (left side) can identify the polymorphisms in these nucleic acids but do not retain linkage information. Employing the reflex process described herein to identify polymorphisms (right side) maintains linkage information.

FIG. **6**: Panel A is a schematic showing expected structures and sizes of nucleic acid species in the reflex process; Panel B is a polyacrylamide gel showing the nucleic acid species produced in the reflex process described in Example 1.

FIG. **7**: Panel A is a schematic showing the structure of the nucleic acid and competitor used in the reflex process; Panel B is a polyacrylamide gel showing the nucleic acid species produced in the reflex process described in Example 1.

FIG. **8** shows a flow chart of a reflex process (left) in which the T7 exonuclease step is optional. The gel on the right shows the resultant product of the reflex process either without the T7 exonuclease step (lane 1) or with the T7 exonuclease step (lane 2).

FIG. **9** shows an exemplary reflex process workflow with indications on the right as to where purification of reaction products is employed (e.g., using Agencourt beads to remove primer oligos).

FIG. **10** shows the starting material (left panel) and the resultant product generated (right panel) using a reflex process without using a T7 exonuclease step (as described in Example II). The reflex site in the starting material is a sequence normally present in the polynucleotide being processed (also called a "non-artificial" reflex site). This figure shows that the 755 base pair starting nucleic acid was processed to the expected 461 base pair product, thus

confirming that a "non-artificial" reflex site is effective in transferring an adapter domain from one location to another in a polynucleotide of interest in a sequence specific manner.

FIG. **11** shows a schematic and results of an experiment in which the reflex process is performed on a single large initial template (a "parent" fragment) to generate five different products ("daughter" products) each having a different region of interest (i.e., daughter products are produced having either region **1**, **2**, **3**, **4** or **5**).

FIG. **12** shows a schematic and results of experiments performed to determine the prevalence of intramolecular rearrangement during the reflex process (as desired) vs. intermolecular rearrangement (MID switching).

FIG. **13** shows a diagram of exemplary workflows for preparing material for and performing the reflex process.

DEFINITIONS

Unless otherwise defined, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. Still, certain elements are defined for the sake of clarity and ease of reference.

Terms and symbols of nucleic acid chemistry, biochemistry, genetics, and molecular biology used herein follow those of standard treatises and texts in the field, e.g. Kornberg and Baker, DNA Replication, Second Edition (W.H. Freeman, New York, 1992); Lehninger, Biochemistry, Second Edition (Worth Publishers, New York, 1975); Strachan and Read, Human Molecular Genetics, Second Edition (Wiley-Liss, New York, 1999); Eckstein, editor, Oligonucleotides and Analogs: A Practical Approach (Oxford University Press, New York, 1991); Gait, editor, Oligonucleotide Synthesis: A Practical Approach (IRL Press, Oxford, 1984); and the like.

"Amplicon" means the product of a polynucleotide amplification reaction. That is, it is a population of polynucleotides, usually double stranded, that are replicated from one or more starting sequences. The one or more starting sequences may be one or more copies of the same sequence, or it may be a mixture of different sequences. Amplicons may be produced by a variety of amplification reactions whose products are multiple replicates of one or more target nucleic acids. Generally, amplification reactions producing amplicons are "template-driven" in that base pairing of reactants, either nucleotides or oligonucleotides, have complements in a template polynucleotide that are required for the creation of reaction products. In one aspect, template-driven reactions are primer extensions with a nucleic acid polymerase or oligonucleotide ligations with a nucleic acid ligase. Such reactions include, but are not limited to, polymerase chain reactions (PCRs), linear polymerase reactions, nucleic acid sequence-based amplification (NASBAs), rolling circle amplifications, and the like, disclosed in the following references that are incorporated herein by reference: Mullis et al, U.S. Pat. Nos. 4,683,195; 4,965,188; 4,683,202; 4,800,159 (PCR); Gelfand et al, U.S. Pat. No. 5,210,015 (real-time PCR with "TAQMAN™" probes); Wittwer et al, U.S. Pat. No. 6,174,670; Kacian et al, U.S. Pat. No. 5,399,491 ("NASBA"); Lizardi, U.S. Pat. No. 5,854,033; Aono et al, Japanese patent publ. JP 4-262799 (rolling circle amplification); and the like. In one aspect, amplicons of the invention are produced by PCRs. An amplification reaction may be a "real-time" amplification if a detection chemistry is available that permits a reaction product to be measured as the amplification reaction progresses, e.g. "real-time PCR" described below, or "real-time NASBA" as described in Leone et al, Nucleic Acids Research, 26: 2150-2155 (1998), and like references. As used herein, the term "amplifying" means performing an amplification reaction. A "reaction mixture" means a solution containing all the necessary reactants for performing a reaction, which may include, but not be limited to, buffering agents to maintain pH at a selected level during a reaction, salts, co-factors, scavengers, and the like.

The term "assessing" includes any form of measurement, and includes determining if an element is present or not. The terms "determining", "measuring", "evaluating", "assessing" and "assaying" are used interchangeably and includes quantitative and qualitative determinations. Assessing may be relative or absolute. "Assessing the presence of" includes determining the amount of something present, and/or determining whether it is present or absent. As used herein, the terms "determining," "measuring," and "assessing," and "assaying" are used interchangeably and include both quantitative and qualitative determinations.

Polynucleotides that are "asymmetrically tagged" have left and right adapter domains that are not identical. This process is referred to generically as attaching adapters asymmetrically or asymmetrically tagging a polynucleotide, e.g., a polynucleotide fragment. Production of polynucleotides having asymmetric adapter termini may be achieved in any convenient manner. Exemplary asymmetric adapters are described in: U.S. Pat. Nos. 5,712,126 and 6,372,434; U.S. Patent Publications 2007/0128624 and 2007/0172839; and PCT publication WO/2009/032167; all of which are incorporated by reference herein in their entirety. In certain embodiments, the asymmetric adapters employed are those described in U.S. patent application Ser. No. 12/432,080, filed on Apr. 29, 2009, incorporated herein by reference in its entirety.

As one example, a user of the subject invention may use an asymmetric adapter to tag polynucleotides. An "asymmetric adapter" is one that, when ligated to both ends of a double stranded nucleic acid fragment, will lead to the production of primer extension or amplification products that have non-identical sequences flanking the genomic insert of interest. The ligation is usually followed by subsequent processing steps so as to generate the non-identical terminal adapter sequences. For example, replication of an asymmetric adapter attached fragment(s) results in polynucleotide products in which there is at least one nucleic acid sequence difference, or nucleotide/nucleoside modification, between the terminal adapter sequences. Attaching adapters asymmetrically to polynucleotides (e.g., polynucleotide fragments) results in polynucleotides that have one or more adapter sequences on one end (e.g., one or more region or domain, e.g., a primer site) that are either not present or have a different nucleic acid sequence as compared to the adapter sequence on the other end. It is noted that an adapter that is termed an "asymmetric adapter" is not necessarily itself structurally asymmetric, nor does the mere act of attaching an asymmetric adapter to a polynucleotide fragment render it immediately asymmetric. Rather, an asymmetric adapter-attached polynucleotide, which has an identical asymmetric adapter at each end, produces replication products (or isolated single stranded polynucleotides) that are asymmetric with respect to the adapter sequences on opposite ends (e.g., after at least one round of amplification/primer extension).

Any convenient asymmetric adapter, or process for attaching adapters asymmetrically, may be employed in practicing the present invention. Exemplary asymmetric adapters are described in: U.S. Pat. Nos. 5,712,126 and

6,372,434; U.S. Patent Publications 2007/0128624 and 2007/0172839; and PCT publication WO/2009/032167; all of which are incorporated by reference herein in their entirety. In certain embodiments, the asymmetric adapters employed are those described in U.S. patent application Ser. No. 12/432,080, filed on Apr. 29, 2009, incorporated herein by reference in its entirety.

"Complementary" or "substantially complementary" refers to the hybridization or base pairing or the formation of a duplex between nucleotides or nucleic acids, such as, for instance, between the two strands of a double stranded DNA molecule or between an oligonucleotide primer and a primer site on a single stranded nucleic acid. Complementary nucleotides are, generally, A and T (or A and U), or C and G. Two single stranded RNA or DNA molecules are said to be substantially complementary when the nucleotides of one strand, optimally aligned and compared and with appropriate nucleotide insertions or deletions, pair with at least about 80% of the nucleotides of the other strand, usually at least about 90% to 95%, and more preferably from about 98 to 100%. Alternatively, substantial complementarity exists when an RNA or DNA strand will hybridize under selective hybridization conditions to its complement. Typically, selective hybridization will occur when there is at least about 65% complementary over a stretch of at least 14 to 25 nucleotides, preferably at least about 75%, more preferably at least about 90% complementary. See, M. Kanehisa Nucleic Acids Res. 12:203 (1984), incorporated herein by reference.

"Duplex" means at least two oligonucleotides and/or polynucleotides that are fully or partially complementary undergo Watson-Crick type base pairing among all or most of their nucleotides so that a stable complex is formed. The terms "annealing" and "hybridization" are used interchangeably to mean the formation of a stable duplex. "Perfectly matched" in reference to a duplex means that the poly- or oligonucleotide strands making up the duplex form a double stranded structure with one another such that every nucleotide in each strand undergoes Watson-Crick base pairing with a nucleotide in the other strand. A stable duplex can include Watson-Crick base pairing and/or non-Watson-Crick base pairing between the strands of the duplex (where base pairing means the forming hydrogen bonds). In certain embodiments, a non-Watson-Crick base pair includes a nucleoside analog, such as deoxyinosine, 2, 6-diaminopurine, PNAs, LNA's and the like. In certain embodiments, a non-Watson-Crick base pair includes a "wobble base", such as deoxyinosine, 8-oxo-dA, 8-oxo-dG and the like, where by "wobble base" is meant a nucleic acid base that can base pair with a first nucleotide base in a complementary nucleic acid strand but that, when employed as a template strand for nucleic acid synthesis, leads to the incorporation of a second, different nucleotide base into the synthesizing strand (wobble bases are described in further detail below). A "mismatch" in a duplex between two oligonucleotides or polynucleotides means that a pair of nucleotides in the duplex fails to undergo Watson-Crick bonding.

"Genetic locus," "locus," or "locus of interest" in reference to a genome or target polynucleotide, means a contiguous sub-region or segment of the genome or target polynucleotide. As used herein, genetic locus, locus, or locus of interest may refer to the position of a nucleotide, a gene or a portion of a gene in a genome, including mitochondrial DNA or other non-chromosomal DNA (e.g., bacterial plasmid), or it may refer to any contiguous portion of genomic sequence whether or not it is within, or associated with, a gene. A genetic locus, locus, or locus of interest can be from a single nucleotide to a segment of a few hundred or a few thousand nucleotides in length or more. In general, a locus of interest will have a reference sequence associated with it (see description of "reference sequence" below).

"Kit" refers to any delivery system for delivering materials or reagents for carrying out a method of the invention. In the context of reaction assays, such delivery systems include systems that allow for the storage, transport, or delivery of reaction reagents (e.g., probes, enzymes, etc. in the appropriate containers) and/or supporting materials (e.g., buffers, written instructions for performing the assay etc.) from one location to another. For example, kits include one or more enclosures (e.g., boxes) containing the relevant reaction reagents and/or supporting materials. Such contents may be delivered to the intended recipient together or separately. For example, a first container may contain an enzyme for use in an assay, while a second container contains probes.

"Ligation" means to form a covalent bond or linkage between the termini of two or more nucleic acids, e.g. oligonucleotides and/or polynucleotides, in a template-driven reaction. The nature of the bond or linkage may vary widely and the ligation may be carried out enzymatically or chemically. As used herein, ligations are usually carried out enzymatically to form a phosphodiester linkage between a 5' carbon of a terminal nucleotide of one oligonucleotide with 3' carbon of another oligonucleotide. A variety of template-driven ligation reactions are described in the following references, which are incorporated by reference: Whiteley et al, U.S. Pat. No. 4,883,750; Letsinger et al, U.S. Pat. No. 5,476,930; Fung et al, U.S. Pat. No. 5,593,826; Kool, U.S. Pat. No. 5,426,180; Landegren et al, U.S. Pat. No. 5,871,921; Xu and Kool, Nucleic Acids Research, 27: 875-881 (1999); Higgins et al, Methods in Enzymology, 68: 50-71 (1979); Engler et al, The Enzymes, 15: 3-29 (1982); and Namsaraev, U.S. patent publication 2004/0110213.

"Multiplex Identifier" (MID) as used herein refers to a tag or combination of tags associated with a polynucleotide whose identity (e.g., the tag DNA sequence) can be used to differentiate polynucleotides in a sample. In certain embodiments, the MID on a polynucleotide is used to identify the source from which the polynucleotide is derived. For example, a nucleic acid sample may be a pool of polynucleotides derived from different sources, (e.g., polynucleotides derived from different individuals, different tissues or cells, or polynucleotides isolated at different times points), where the polynucleotides from each different source are tagged with a unique MID. As such, a MID provides a correlation between a polynucleotide and its source. In certain embodiments, MIDs are employed to uniquely tag each individual polynucleotide in a sample. Identification of the number of unique MIDs in a sample can provide a readout of how many individual polynucleotides are present in the sample (or from how many original polynucleotides a manipulated polynucleotide sample was derived; see, e.g., U.S. Pat. No. 7,537,897, issued on May 26, 2009, incorporated herein by reference in its entirety). MIDs can range in length from 2 to 100 nucleotide bases or more and may include multiple subunits, where each different MID has a distinct identity and/or order of subunits. Exemplary nucleic acid tags that find use as MIDs are described in U.S. Pat. No. 7,544,473, issued on Jun. 6, 2009, and titled "Nucleic Acid Analysis Using Sequence Tokens", as well as U.S. Pat. No. 7,393,665, issued on Jul. 1, 2008, and titled "Methods and Compositions for Tagging and Identifying Polynucleotides", both of which are incorporated herein by reference in their entirety for their description of nucleic acid tags and their use in

identifying polynucleotides. In certain embodiments, a set of MIDs employed to tag a plurality of samples need not have any particular common property (e.g., Tm, length, base composition, etc.), as the methods described herein can accommodate a wide variety of unique MID sets. It is emphasized here that MIDs need only be unique within a given experiment. Thus, the same MID may be used to tag a different sample being processed in a different experiment. In addition, in certain experiments, a user may use the same MID to tag a subset of different samples within the same experiment. For example, all samples derived from individuals having a specific phenotype may be tagged with the same MID, e.g., all samples derived from control (or wild-type) subjects can be tagged with a first MID while subjects having a disease condition can be tagged with a second MID (different than the first MID). As another example, it may be desirable to tag different samples derived from the same source with different MIDs (e.g., samples derived over time or derived from different sites within a tissue). Further, MIDs can be generated in a variety of different ways, e.g., by a combinatorial tagging approach in which one MID is attached by ligation and a second MID is attached by primer extension. Thus, MIDs can be designed and implemented in a variety of different ways to track polynucleotide fragments during processing and analysis, and thus no limitation in this regard is intended.

"Nucleoside" as used herein includes the natural nucleosides, including 2'-deoxy and 2'-hydroxyl forms, e.g. as described in Kornberg and Baker, DNA Replication, 2nd Ed. (Freeman, San Francisco, 1992). "Analogs" in reference to nucleosides includes synthetic nucleosides having modified base moieties and/or modified sugar moieties, e.g. described by Scheit, Nucleotide Analogs (John Wiley, New York, 1980); Uhlman and Peyman, Chemical Reviews, 90: 543-584 (1990), or the like, with the proviso that they are capable of specific hybridization. Such analogs include synthetic nucleosides designed to enhance binding properties, reduce complexity, increase specificity, and the like. Polynucleotides comprising analogs with enhanced hybridization or nuclease resistance properties are described in Uhlman and Peyman (cited above); Crooke et al, Exp. Opin. Ther. Patents, 6: 855-870 (1996); Mesmaeker et al, Current Opinion in Structual Biology, 5: 343-355 (1995); and the like. Exemplary types of polynucleotides that are capable of enhancing duplex stability include oligonucleotide N3'→P5' phosphoramidates (referred to herein as "amidates"), peptide nucleic acids (referred to herein as "PNAs"), oligo-2'-O-alkylribonucleotides, polynucleotides containing C-5 propynylpyrimidines, locked nucleic acids ("LNAs"), and like compounds. Such oligonucleotides are either available commercially or may be synthesized using methods described in the literature.

"Polymerase chain reaction," or "PCR," means a reaction for the in vitro amplification of specific DNA sequences by the simultaneous primer extension of complementary strands of DNA. In other words, PCR is a reaction for making multiple copies or replicates of a target nucleic acid flanked by primer sites, such reaction comprising one or more repetitions of the following steps: (i) denaturing the target nucleic acid, (ii) annealing primers to the primer sites, and (iii) extending the primers by a nucleic acid polymerase in the presence of nucleoside triphosphates. Usually, the reaction is cycled through different temperatures optimized for each step in a thermal cycler instrument. Particular temperatures, durations at each step, and rates of change between steps depend on many factors well-known to those of ordinary skill in the art, e.g. exemplified by the references: McPherson et al, editors, PCR: A Practical Approach and PCR2: A Practical Approach (IRL Press, Oxford, 1991 and 1995, respectively). For example, in a conventional PCR using Taq DNA polymerase, a double stranded target nucleic acid may be denatured at a temperature >90° C., primers annealed at a temperature in the range 50-75° C., and primers extended at a temperature in the range 72-78° C. The term "PCR" encompasses derivative forms of the reaction, including but not limited to, RT-PCR, real-time PCR, nested PCR, quantitative PCR, multiplexed PCR, and the like. Reaction volumes range from a few hundred nanoliters, e.g. 200 nL, to a few hundred μL, e.g. 200 μL. "Reverse transcription PCR," or "RT-PCR," means a PCR that is preceded by a reverse transcription reaction that converts a target RNA to a complementary single stranded DNA, which is then amplified, e.g. Tecott et al, U.S. Pat. No. 5,168,038, which patent is incorporated herein by reference. "Real-time PCR" means a PCR for which the amount of reaction product, i.e. amplicon, is monitored as the reaction proceeds. There are many forms of real-time PCR that differ mainly in the detection chemistries used for monitoring the reaction product, e.g. Gelfand et al, U.S. Pat. No. 5,210,015 ("TAQMAN™"); Wittwer et al, U.S. Pat. Nos. 6,174,670 and 6,569,627 (intercalating dyes); Tyagi et al, U.S. Pat. No. 5,925,517 (molecular beacons); which patents are incorporated herein by reference. Detection chemistries for real-time PCR are reviewed in Mackay et al, Nucleic Acids Research, 30: 1292-1305 (2002), which is also incorporated herein by reference. "Nested PCR" means a two-stage PCR wherein the amplicon of a first PCR becomes the sample for a second PCR using a new set of primers, at least one of which binds to an interior location of the first amplicon. As used herein, "initial primers" in reference to a nested amplification reaction mean the primers used to generate a first amplicon, and "secondary primers" mean the one or more primers used to generate a second, or nested, amplicon. "Multiplexed PCR" means a PCR wherein multiple target sequences (or a single target sequence and one or more reference sequences) are simultaneously carried out in the same reaction mixture, e.g. Bernard et al, Anal. Biochem., 273: 221-228 (1999)(two-color real-time PCR). Usually, distinct sets of primers are employed for each sequence being amplified.

"Quantitative PCR" means a PCR designed to measure the abundance of one or more specific target sequences in a sample or specimen. Quantitative PCR includes both absolute quantitation and relative quantitation of such target sequences. Quantitative measurements are made using one or more reference sequences that may be assayed separately or together with a target sequence. The reference sequence may be endogenous or exogenous to a sample or specimen, and in the latter case, may comprise one or more competitor templates. Typical endogenous reference sequences include segments of transcripts of the following genes: β-actin, GAPDH, β_2-microglobulin, ribosomal RNA, and the like. Techniques for quantitative PCR are well-known to those of ordinary skill in the art, as exemplified in the following references that are incorporated by reference: Freeman et al, Biotechniques, 26: 112-126 (1999); Becker-Andre et al, Nucleic Acids Research, 17: 9437-9447 (1989); Zimmerman et al, Biotechniques, 21: 268-279 (1996); Diviacco et al, Gene, 122: 3013-3020 (1992); Becker-Andre et al, Nucleic Acids Research, 17: 9437-9446 (1989); and the like.

"Polynucleotide" or "oligonucleotide" is used interchangeably and each means a linear polymer of nucleotide monomers. Monomers making up polynucleotides and oligonucleotides are capable of specifically binding to a natural

polynucleotide by way of a regular pattern of monomer-to-monomer interactions, such as Watson-Crick type of base pairing, base stacking, Hoogsteen or reverse Hoogsteen types of base pairing, wobble base pairing, or the like. As described in detail below, by "wobble base" is meant a nucleic acid base that can base pair with a first nucleotide base in a complementary nucleic acid strand but that, when employed as a template strand for nucleic acid synthesis, leads to the incorporation of a second, different nucleotide base into the synthesizing strand. Such monomers and their internucleosidic linkages may be naturally occurring or may be analogs thereof, e.g. naturally occurring or non-naturally occurring analogs. Non-naturally occurring analogs may include peptide nucleic acids (PNAs, e.g., as described in U.S. Pat. No. 5,539,082, incorporated herein by reference), locked nucleic acids (LNAs, e.g., as described in U.S. Pat. No. 6,670,461, incorporated herein by reference), phosphorothioate internucleosidic linkages, bases containing linking groups permitting the attachment of labels, such as fluorophores, or haptens, and the like. Whenever the use of an oligonucleotide or polynucleotide requires enzymatic processing, such as extension by a polymerase, ligation by a ligase, or the like, one of ordinary skill would understand that oligonucleotides or polynucleotides in those instances would not contain certain analogs of internucleosidic linkages, sugar moieties, or bases at any or some positions. Polynucleotides typically range in size from a few monomeric units, e.g. 5-40, when they are usually referred to as "oligonucleotides," to several thousand monomeric units. Whenever a polynucleotide or oligonucleotide is represented by a sequence of letters (upper or lower case), such as "ATGCCTG," it will be understood that the nucleotides are in 5'→3' order from left to right and that "A" denotes deoxyadenosine, "C" denotes deoxycytidine, "G" denotes deoxyguanosine, and "T" denotes thymidine, "I" denotes deoxyinosine, "U" denotes uridine, unless otherwise indicated or obvious from context. Unless otherwise noted the terminology and atom numbering conventions will follow those disclosed in Strachan and Read, Human Molecular Genetics 2 (Wiley-Liss, New York, 1999). Usually polynucleotides comprise the four natural nucleosides (e.g. deoxyadenosine, deoxycytidine, deoxyguanosine, deoxythymidine for DNA or their ribose counterparts for RNA) linked by phosphodiester linkages; however, they may also comprise non-natural nucleotide analogs, e.g. including modified bases, sugars, or internucleosidic linkages. It is clear to those skilled in the art that where an enzyme has specific oligonucleotide or polynucleotide substrate requirements for activity, e.g. single stranded DNA, RNA/DNA duplex, or the like, then selection of appropriate composition for the oligonucleotide or polynucleotide substrates is well within the knowledge of one of ordinary skill, especially with guidance from treatises, such as Sambrook et al, Molecular Cloning, Second Edition (Cold Spring Harbor Laboratory, New York, 1989), and like references.

"Primer" means an oligonucleotide, either natural or synthetic, that is capable, upon forming a duplex with a polynucleotide template, of acting as a point of initiation of nucleic acid synthesis and being extended from its 3' end along the template so that an extended duplex is formed. The sequence of nucleotides added during the extension process is determined by the sequence of the template polynucleotide. Usually primers are extended by a DNA polymerase. Primers are generally of a length compatible with their use in synthesis of primer extension products, and are usually are in the range of between 8 to 100 nucleotides in length, such as 10 to 75, 15 to 60, 15 to 40, 18 to 30, 20 to 40, 21 to 50, 22 to 45, 25 to 40, and so on, more typically in the range of between 18-40, 20-35, 21-30 nucleotides long, and any length between the stated ranges. Typical primers can be in the range of between 10-50 nucleotides long, such as 15-45, 18-40, 20-30, 21-25 and so on, and any length between the stated ranges. In some embodiments, the primers are usually not more than about 10, 12, 15, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 35, 40, 45, 50, 55, 60, 65, or 70 nucleotides in length.

Primers are usually single-stranded for maximum efficiency in amplification, but may alternatively be double-stranded. If double-stranded, the primer is usually first treated to separate its strands before being used to prepare extension products. This denaturation step is typically affected by heat, but may alternatively be carried out using alkali, followed by neutralization. Thus, a "primer" is complementary to a template, and complexes by hydrogen bonding or hybridization with the template to give a primer/template complex for initiation of synthesis by a polymerase, which is extended by the addition of covalently bonded bases linked at its 3' end complementary to the template in the process of DNA synthesis.

A "primer pair" as used herein refers to first and second primers having nucleic acid sequence suitable for nucleic acid-based amplification of a target nucleic acid. Such primer pairs generally include a first primer having a sequence that is the same or similar to that of a first portion of a target nucleic acid, and a second primer having a sequence that is complementary to a second portion of a target nucleic acid to provide for amplification of the target nucleic acid or a fragment thereof. Reference to "first" and "second" primers herein is arbitrary, unless specifically indicated otherwise. For example, the first primer can be designed as a "forward primer" (which initiates nucleic acid synthesis from a 5' end of the target nucleic acid) or as a "reverse primer" (which initiates nucleic acid synthesis from a 5' end of the extension product produced from synthesis initiated from the forward primer). Likewise, the second primer can be designed as a forward primer or a reverse primer.

"Primer site" (e.g., a sequencing primer site, and amplification primer site, etc.) as used herein refers to a domain in a polynucleotide that includes the sequence of a primer (e.g., a sequencing primer) and/or the complementary sequence of a primer. When present in single stranded form (e.g., in a single stranded polynucleotide), a primer site can be either the identical sequence of a primer or the complementary sequence of a primer. When present in double stranded form, a primer site contains the sequence of a primer hybridized to the complementary sequence of the primer. Thus, a primer site is a region of a polynucleotide that is either identical to or complementary to the sequence of a primer (when in a single stranded form) or a double stranded region formed between a primer sequence and its complement. Primer sites may be present in an adapter attached to a polynucleotide. The specific orientation of a primer site can be inferred by those of ordinary skill in the art from the structural features of the relevant polynucleotide and/or context in which it is used.

"Readout" means a parameter, or parameters, which are measured and/or detected that can be converted to a number or value. In some contexts, readout may refer to an actual numerical representation of such collected or recorded data. For example, a readout of fluorescent intensity signals from a microarray is the address and fluorescence intensity of a signal being generated at each hybridization site of the microarray; thus, such a readout may be registered or stored

in various ways, for example, as an image of the microarray, as a table of numbers, or the like.

"Reflex site", "reflex sequence" and equivalents are used to indicate sequences in a polynucleotide that are employed to move a domain intramolecularly from its initial location to a different location in the polynucleotide. The sequence of a reflex site can be added to a polynucleotide of interest (e.g., present in an adapter ligated to the polynucleotide), be based on a sequence naturally present within the polynucleotide of interest (e.g., a genomic sequence in the polynucleotide), or a combination of both. The reflex sequence is chosen so as to be distinct from other sequences in the polynucleotide (i.e., with little sequence homology to other sequences likely to be present in the polynucleotide, e.g., genomic or sub-genomic sequences to be processed). As such, a reflex sequence should be selected so as to not hybridize to any sequence except its complement under the conditions employed in the reflex processes herein described. As described later in this application, the complement to the reflex sequence is inserted on the same strand of the polynucleotide (e.g., the same strand of a double-stranded polynucleotide or on the same single stranded polynucleotide) in a particular location so as to facilitate an intramolecular binding event on such particular strand. Reflex sequences employed in the reflex process described herein can thus have a wide range of lengths and sequences. Reflex sequences may range from 5 to 200 nucleotide bases in length.

"Solid support", "support", and "solid phase support" are used interchangeably and refer to a material or group of materials having a rigid or semi-rigid surface or surfaces. In many embodiments, at least one surface of the solid support will be substantially flat, although in some embodiments it may be desirable to physically separate synthesis regions for different compounds with, for example, wells, raised regions, pins, etched trenches, or the like. According to other embodiments, the solid support(s) will take the form of beads, resins, gels, microspheres, or other geometric configurations. Microarrays usually comprise at least one planar solid phase support, such as a glass microscope slide.

"Specific" or "specificity" in reference to the binding of one molecule to another molecule, such as a labeled target sequence for a probe, means the recognition, contact, and formation of a stable complex between the two molecules, together with substantially less recognition, contact, or complex formation of that molecule with other molecules. In one aspect, "specific" in reference to the binding of a first molecule to a second molecule means that to the extent the first molecule recognizes and forms a complex with another molecule in a reaction or sample, it forms the largest number of the complexes with the second molecule. Preferably, this largest number is at least fifty percent. Generally, molecules involved in a specific binding event have areas on their surfaces or in cavities giving rise to specific recognition between the molecules binding to each other. Examples of specific binding include antibody-antigen interactions, enzyme-substrate interactions, formation of duplexes or triplexes among polynucleotides and/or oligonucleotides, biotin-avidin or biotin-streptavidin interactions, receptor-ligand interactions, and the like. As used herein, "contact" in reference to specificity or specific binding means two molecules are close enough that weak noncovalent chemical interactions, such as Van der Waal forces, hydrogen bonding, base-stacking interactions, ionic and hydrophobic interactions, and the like, dominate the interaction of the molecules.

As used herein, the term "T_m" is used in reference to the "melting temperature." The melting temperature is the temperature (e.g., as measured in ° C.) at which a population of double-stranded nucleic acid molecules becomes half dissociated into single strands. Several equations for calculating the T_m of nucleic acids are known in the art (see e.g., Anderson and Young, Quantitative Filter Hybridization, in Nucleic Acid Hybridization (1985). Other references (e.g., Allawi, H. T. & SantaLucia, J., Jr., Biochemistry 36, 10581-94 (1997)) include alternative methods of computation which take structural and environmental, as well as sequence characteristics into account for the calculation of T_m.

"Sample" means a quantity of material from a biological, environmental, medical, or patient source in which detection, measurement, or labeling of target nucleic acids is sought. On the one hand it is meant to include a specimen or culture (e.g., microbiological cultures). On the other hand, it is meant to include both biological and environmental samples. A sample may include a specimen of synthetic origin. Biological samples may be animal, including human, fluid, solid (e.g., stool) or tissue, as well as liquid and solid food and feed products and ingredients such as dairy items, vegetables, meat and meat by-products, and waste. Biological samples may include materials taken from a patient including, but not limited to cultures, blood, saliva, cerebral spinal fluid, pleural fluid, milk, lymph, sputum, semen, needle aspirates, and the like. Biological samples may be obtained from all of the various families of domestic animals, as well as feral or wild animals, including, but not limited to, such animals as ungulates, bear, fish, rodents, etc. Environmental samples include environmental material such as surface matter, soil, water and industrial samples, as well as samples obtained from food and dairy processing instruments, apparatus, equipment, utensils, disposable and non-disposable items. These examples are not to be construed as limiting the sample types applicable to the present invention.

The terms "upstream" and "downstream" in describing nucleic acid molecule orientation and/or polymerization are used herein as understood by one of skill in the art. As such, "downstream" generally means proceeding in the 5' to 3' direction, i.e., the direction in which a nucleotide polymerase normally extends a sequence, and "upstream" generally means the converse. For example, a first primer that hybridizes "upstream" of a second primer on the same target nucleic acid molecule is located on the 5' side of the second primer (and thus nucleic acid polymerization from the first primer proceeds towards the second primer).

It is further noted that the claims may be drafted to exclude any optional element. As such, this statement is intended to serve as antecedent basis for use of such exclusive terminology as "solely", "only" and the like in connection with the recitation of claim elements, or the use of a "negative" limitation.

DETAILED DESCRIPTION OF THE INVENTION

The invention is drawn to compositions and methods for intramolecular nucleic acid rearrangement that find use in various applications of genetic analysis, including sequencing, as well as general molecular biological manipulations of polynucleotide structures.

Before the present invention is described, it is to be understood that this invention is not limited to particular embodiments described, as such may, of course, vary. It is also to be understood that the terminology used herein is for the purpose of describing particular embodiments only, and is not intended to be limiting, since the scope of the present invention will be limited only by the appended claims.

Where a range of values is provided, it is understood that each intervening value, to the tenth of the unit of the lower limit unless the context clearly dictates otherwise, between the upper and lower limits of that range is also specifically disclosed. Each smaller range between any stated value or intervening value in a stated range and any other stated or intervening value in that stated range is encompassed within the invention. The upper and lower limits of these smaller ranges may independently be included or excluded in the range, and each range where either, neither or both limits are included in the smaller ranges is also encompassed within the invention, subject to any specifically excluded limit in the stated range. Where the stated range includes one or both of the limits, ranges excluding either or both of those included limits are also included in the invention.

Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. Although any methods and materials similar or equivalent to those described herein can be used in the practice or testing of the present invention, some potential and preferred methods and materials are now described. All publications mentioned herein are incorporated herein by reference to disclose and describe the methods and/or materials in connection with which the publications are cited. It is understood that the present disclosure supersedes any disclosure of an incorporated publication to the extent there is a contradiction.

It must be noted that as used herein and in the appended claims, the singular forms "a", "an", and "the" include plural referents unless the context clearly dictates otherwise. Thus, for example, reference to "a nucleic acid" includes a plurality of such nucleic acids and reference to "the compound" includes reference to one or more compounds and equivalents thereof known to those skilled in the art, and so forth.

The practice of the present invention may employ, unless otherwise indicated, conventional techniques and descriptions of organic chemistry, polymer technology, molecular biology (including recombinant techniques), cell biology, biochemistry, and immunology, which are within the skill of the art. Such conventional techniques include polymer array synthesis, hybridization, ligation, and detection of hybridization using a label. Specific illustrations of suitable techniques can be had by reference to the example herein below. However, other equivalent conventional procedures can, of course, also be used. Such conventional techniques and descriptions can be found in standard laboratory manuals such as *Genome Analysis: A Laboratory Manual Series* (Vols. I-IV), *Using Antibodies: A Laboratory Manual, Cells: A Laboratory Manual, PCR Primer: A Laboratory Manual, and Molecular Cloning: A Laboratory Manual* (all from Cold Spring Harbor Laboratory Press), Stryer, L. (1995) *Biochemistry* (4th Ed.) Freeman, New York, Gait, "*Oligonucleotide Synthesis: A Practical Approach*" 1984, IRL Press, London, Nelson and Cox (2000), Lehninger, A., *Principles of Biochemistry* 3rd Ed., W. H. Freeman Pub., New York, N.Y. and Berg et al. (2002) *Biochemistry,* 5th Ed., W. H. Freeman Pub., New York, N.Y., all of which are herein incorporated in their entirety by reference for all purposes.

The publications discussed herein are provided solely for their disclosure prior to the filing date of the present application. Nothing herein is to be construed as an admission that the present invention is not entitled to antedate such publication by virtue of prior invention. Further, the dates of publication provided may be different from the actual publication dates which may need to be independently confirmed.

As summarized above, aspects of the present invention are drawn to the use of a 'reflex' sequence present in a polynucleotide (e.g., in an adapter structure of the polynucleotide, in a genomic region of the polynucleotide, or a combination of both) to move a domain of the polynucleotide intra-molecularly from a first location to a second location. The reflex process described herein finds use in any number of applications, e.g., placing functional elements of a polynucleotide (e.g., sequencing primer sites and/or MID tags) into proximity to a desired sub-region of interest.

Nucleic Acids

The reflex process (as described in detail below) can be employed for the manipulation and analysis of nucleic acid sequences of interest from virtually any nucleic acid source, including but not limited to genomic DNA, complementary DNA (cDNA), RNA (e.g., messenger RNA, ribosomal RNA, short interfering RNA, microRNA, etc.), plasmid DNA, mitochondrial DNA, synthetic DNA, etc. Furthermore, any organism, organic material or nucleic acid-containing substance can be used as a source of nucleic acids to be processed in accordance with the present invention including, but not limited to, plants, animals (e.g., reptiles, mammals, insects, worms, fish, etc.), tissue samples, bacteria, fungi (e.g., yeast), phage, viruses, cadaveric tissue, archaeological/ancient samples, etc. In certain embodiments, the nucleic acids in the nucleic acid sample are derived from a mammal, where in certain embodiments the mammal is a human.

In certain embodiments, the nucleic acid sequences are enriched prior to the reflex sequence process. By enriched is meant that the nucleic acid is subjected to a process that reduces the complexity of the nucleic acids, generally by increasing the relative concentration of particular nucleic acid species in the sample (e.g., having a specific locus of interest, including a specific nucleic acid sequence, lacking a locus or sequence, being within a specific size range, etc.). There are a wide variety of ways to enrich nucleic acids having a specific characteristic(s) or sequence, and as such any convenient method to accomplish this may be employed. The enrichment (or complexity reduction) can take place at any of a number of steps in the process, and will be determined by the desires of the user. For example, enrichment can take place in individual parental samples (e.g., untagged nucleic acids prior to adaptor ligation) or in multiplexed samples (e.g., nucleic acids tagged with primer sites, MID and/or reflex sequences and pooled; MID are described in further detail below).

In certain embodiments, nucleic acids in the nucleic acid sample are amplified prior to analysis. In certain of these embodiments, the amplification reaction also serves to enrich a starting nucleic acid sample for a sequence or locus of interest. For example, a starting nucleic acid sample can be subjected to a polymerase chain reaction (PCR) that amplifies one or more region of interest. In certain embodiments, the amplification reaction is an exponential amplification reaction, whereas in certain other embodiments, the amplification reaction is a linear amplification reaction. Any convenient method for performing amplification reactions on a starting nucleic acid sample can be used in practicing the subject invention. In certain embodiments, the nucleic acid polymerase employed in the amplification reaction is a polymerase that has proofreading capability (e.g., phi29 DNA Polymerase, *Thermococcus litoralis* DNA polymerase, *Pyrococcus furiosus* DNA polymerase, etc.).

In certain embodiments, the nucleic acid sample being analyzed is derived from a single source (e.g., a single organism, virus, tissue, cell, subject, etc.), whereas in other

embodiments, the nucleic acid sample is a pool of nucleic acids extracted from a plurality of sources (e.g., a pool of nucleic acids from a plurality of organisms, tissues, cells, subjects, etc.), where by "plurality" is meant two or more. As such, in certain embodiments, a nucleic acid sample can contain nucleic acids from 2 or more sources, 3 or more sources, 5 or more sources, 10 or more sources, 50 or more sources, 100 or more sources, 500 or more sources, 1000 or more sources, 5000 or more sources, up to and including about 10,000 or more sources.

In certain embodiments, nucleic acid fragments that are to be pooled with nucleic acid fragments derived from a plurality of sources (e.g., a plurality of organisms, tissues, cells, subjects, etc.), where by "plurality" is meant two or more. In such embodiments, the nucleic acids derived from each source includes a multiplex identifier (MID) such that the source from which the each tagged nucleic acid fragment was derived can be determined. In such embodiments, each nucleic acid sample source is correlated with a unique MID, where by unique MID is meant that each different MID employed can be differentiated from every other MID employed by virtue of at least one characteristic, e.g., the nucleic acid sequence of the MID. Any type of MID can be used, including but not limited to those described in co-pending U.S. patent application Ser. No. 11/656,746, filed on Jan. 22, 2007, and titled "Nucleic Acid Analysis Using Sequence Tokens", as well as U.S. Pat. No. 7,393,665, issued on Jul. 1, 2008, and titled "Methods and Compositions for Tagging and Identifying Polynucleotides", both of which are incorporated herein by reference in their entirety for their description of nucleic acid tags and their use in identifying polynucleotides. In certain embodiments, a set of MIDs employed to tag a plurality of samples need not have any particular common property (e.g., T_m, length, base composition, etc.), as the asymmetric tagging methods (and many tag readout methods, including but not limited to sequencing of the tag or measuring the length of the tag) can accommodate a wide variety of unique MID sets.

In certain embodiments, each individual polynucleotide (e.g., double-stranded or single-stranded, as appropriate to the methodological details employed) in a sample to be analyzed is tagged with a unique MID so that the fate of each polynucleotide can be tracked in subsequent processes (where, as noted above, unique MID is meant to indicate that each different MID employed can be differentiated from every other MID employed by virtue of at least one characteristic, e.g., the nucleic acid sequence of the MID). For example (and as described below), having each nucleic acid tagged with a unique MID allows analysis of the sequence of each individual nucleic acid using the reflex sequence methods described herein. This allows the linkage of sequence information for large nucleic acid fragments that cannot be sequenced in a single sequencing run.

Reflex Sequence Process

As summarized above, aspects of the present invention include methods and compositions for moving a domain in a polynucleotide from a first location to a second location in the polynucleotide. An exemplary embodiment is shown in FIG. **1**A.

FIG. **1**A shows a single stranded polynucleotide **100** comprising, in a 5' to 3' orientation, a first domain (**102**; the domain to be moved); a reflex sequence **104**; a nucleic acid sequence **106** having a site distal to the first domain (Site A), and a complement of the reflex sequence **108** (positioned at the 3' terminus of the polynucleotide). The steps of the reflex method described below will move the first domain into closer proximity to Site A. It is noted here that the prime designation in FIG. **1**A denotes a complementary sequence of a domain. For example, First Domain' is the complement of the First Domain.

In Step **1**, the reflex sequence and its complement in the polynucleotide are annealed intramolecularly to form polynucleotide structure **112**, with the polynucleotide folding back on itself and hybridizing to form a region of complementarity (i.e., double stranded reflex/reflex' region). In this configuration, the 3' end of the complement of the reflex sequence can serve as a nucleic acid synthesis priming site. Nucleic acid synthesis from this site is then performed in extension Step **2** producing a complement of the first domain at the 3' end of the nucleic acid extension (shown in polynucleotide **114**; extension is indicated with dotted arrow labeled "extend").

Denaturation of polynucleotide **114** (e.g., by heat) generates linear single stranded polynucleotide **116**. As shown in FIG. **1**, resultant polynucleotide **116** contains a complement of the first domain at a position proximal to Site A (i.e., separated by only the complement of the reflex sequence). This resultant polynucleotide may be used for any subsequent analysis or processing steps as desired by the user (e.g., sequencing, as a template for amplification (linear, PCR, etc.), sequence specific extraction, etc.).

In alternative embodiments, the first domain and reflex sequence are removed from the 5' end of the double-stranded region of polynucleotide **114** (shown in polynucleotide **118**; removal is shown in the dotted arrow labeled "remove"). Removal of this region may be accomplished by any convenient method, including, but not limited to, treatment (under appropriate incubation conditions) of polynucleotide structure **114** with T7 exonuclease or by treatment with Lambda exonuclease; the Lambda exonuclease can be employed so long as the 5' end of the polynucleotide is phosphorylated. If the region is removed enzymatically, resultant polynucleotide **118** is used in place of polynucleotide **116** in subsequent steps (e.g., copying to reverse polarity).

In certain embodiments, polynucleotide **116** or **118** is used as a template to produce a double stranded polynucleotide, for example by performing a nucleic acid synthesis reaction with a primer that primes in the complement of the first domain. This step is sometimes referred to as copying to reverse polarity of a single stranded polynucleotide, and in some instances, the double-stranded intermediate product of this copying is not shown (see, e.g., FIG. **3**). For example, copying to reverse the polarity of polynucleotide **116** results in single-stranded polynucleotide **120** having, in a 5' to 3' orientation, the first domain (**122**); the reflex sequence (**124**); the complement of polynucleotide **106** (oriented with the complement of Site A (Site A'; **126**) proximal to the reflex sequence); the complement of the reflex sequence (**128**); and the complement of the first domain (**130**).

In certain embodiments, the first domain in the polynucleotide comprises one or more elements that find use in one or more subsequent processing or analysis steps. Such sequences include, but are not limited to, restriction enzyme sites, PCR primer sites, linear amplification primer sites, reverse transcription primer sites, RNA polymerase promoter sites (such as for T7, T3 or SP6 RNA polymerase), MID tags, sequencing primer sites, etc. Any convenient element can be included in the first domain and, in certain embodiments, is determined by the desires of the user of the methods described herein.

As an exemplary embodiment, suppose we want to sequence a specific polynucleotide region from multiple genomes in a pooled sample where the polynucleotide

region is too long to sequence in a single reaction. For example, sequencing a polynucleotide region that is 2 kilobases or more in length using Roche 454 (Branford, Conn.) technology, in which the length of a single sequencing run is about 400 bases. In this scenario, we can design a set of left hand primers (A_n) and right hand primers (B_n) specific for the polynucleotide region that are positioned in such a way that we can obtain direct sequences of all parts of the insert, as shown in FIG. **1**B. Note that the polynucleotide shown in FIG. **1**B (**140**) has a domain (**142**) containing a primer site and an MID denoting from which original sample(s) the polynucleotide is derived. Site **142** thus represents an example of a First Domain site such identified as **122** in the FIG. **1**A. The polynucleotide also includes a reflex site (**144**), which can be part of the polynucleotide region itself (e.g., a genomic sequence), added in a ligated adapter domain along with the primer site and the MID (an artificial sequence), or a combination of both (a sequence spanning the adapter/polynucleotide junction).

It is noted here that polynucleotide **140** can be categorized as a precursor to polynucleotide **100** in FIG. **1**A, as it does not include a 3' reflex sequence complementary to the reflex site (domain **108** in FIG. **1**A). As detailed below, polynucleotide **140** can be converted to a polynucleotide having the structural configuration of polynucleotide **100**, a polynucleotide suitable as a substrate for the reflex process described herein (e.g., by primer extension using a B_n primer and reversal of polarity).

In an exemplary embodiment, each A_n-B_n primer pair defines a nucleic acid region that is approximately 400 bases in length or less. This size range is within the single-sequencing run read length of the current Roche 454 sequencing platform; a different size range for the defined nucleic acid region may be utilized for a different sequencing platform. Thus, each product from each reflex process can be sequenced in a single run. It is noted here that primer pairs as shown in FIG. **1**B can be used to define regions **1** to **5** shown in FIG. **3** (described in further detail below).

In certain embodiments, to obtain the first part of the sequence of the polynucleotide region (i.e., in the original structure, that part of the polynucleotide closest to the first domain), we only need a right hand primer (e.g., B_0) and we do not need to transfer the MID as it is within reach of this sequencing primer (i.e., the MID is within 400 bases of sequencing primer B_0). All other B_n primers have the reflex sequence added to their 5' ends ("R" element shown on B primers) so that they read 5' reflex-B_n. However, in certain embodiments, the B_0 primer does include the reflex sequence and is used in the reflex process (along with a corresponding A_0 primer) as detailed below.

As described above, we obtain a single stranded polynucleotide having, in the 5' to 3' orientation, a primer site (e.g., for Roche 454 sequencing), an MID, a reflex sequence and the polynucleotide to be sequenced. Numerous methods for obtaining single-stranded polynucleotides of interest have been described and are known in the art, including in U.S. Pat. No. 7,217,522, issued on May 15, 2007; U.S. patent application Ser. No. 11/377,462, filed on Mar. 16, 2006; and U.S. patent application Ser. No. 12/432,080, filed on Apr. 29, 2009; each of which is incorporated by reference herein in their entirety. For example, a single stranded product can be produced using linear amplification with a primer specific for the primer site of the template. In certain embodiments, the primer includes a binding moiety to facilitate isolation of the single stranded nucleic acid of interest, e.g., to immobilize the top strand on a binding partner of the binding moiety immobilized on a solid support. Removal of a hybridized, non-biotinylated strand by denaturation using heat or high pH (or any other convenient method) serves to isolate the biotinylated strand. Binding moieties and their corresponding binding partners are sometimes referred to herein as binding partner pairs. Any convenient binding partner pairs may be used, including but not limited to biotin/avidin (or streptavidin), antigen/antibody pairs, etc.

It is noted here that while the figures and description of the reflex process provided herein depict manipulations with regard to a single stranded polynucleotide, it is not necessarily required that the single stranded polynucleotide described or depicted in the figures be present in the sample in an isolated form (i.e., isolated from its complementary strand). In other words, double stranded polynucleotides may be used where only one strand is described/depicted, which will generally be determined by the user.

The implementation of a single strand isolation step using the methods described above or variations thereof (or any other convenient single strand isolation step) will generally be based on the desires of the user. One example of isolating single stranded polynucleotides is shown in FIG. **2**. In this Figure, a starting double stranded template (with 5' to 3' orientation shown as an arrow) is denatured and primed with a biotinylated synthesis primer specific for the primer site. After extension of the primer (i.e., nucleic acid synthesis), the sample is contacted with a solid support having streptavidin bound to it. The biotin moiety (i.e., the binding partner of streptavidin) on the extended strands will bind to the solid-phase streptavidin. Denaturation and washing is then performed to remove all non-biotinylated polynucleotide strands. If desired, the bound polynucleotide, which can be used in subsequent reflex process steps (e.g., as a template for B_n primer extension reactions), may be eluted from the streptavidin support. Alternatively, the bound polynucleotide may be employed in subsequent steps of the desired process while still bound to the solid support (e.g., in solid phase extension reactions using B_n primers). This process, with minor variations depending on the template being used and the identity of the desired single stranded polynucleotide, may be employed at any of a number of steps in which a single stranded product is to be isolated. It is noted that in certain embodiments, substrate bound biotinylated polynucleotide can be used to produce and isolate non-biotinylated single stranded products (i.e., by eluting the non-biotinylated products while leaving the biotinylated templates bound to the streptavidin on the solid support). Thus, the specifics of how binding partners are used to isolate single stranded polynucleotides of interest will vary depending on experimental design parameters.

Additional single-stranded isolation/production methods include asymmetric PCR, strand-specific enzymatic degradation, and the use of in-vitro transcription followed by reverse transcriptase (IVT-RT) with subsequent destruction of the RNA strand. As noted above, any convenient single stranded production/isolation method may be employed.

To the single stranded polynucleotide shown in FIG. **1**B we anneal one of the B_n primers having the appended reflex sequence, denoted with a capital "R" (e.g., B_1) and extend the primer under nucleic acid synthesis conditions to produce a copy of the polynucleotide that has a reflex sequence at its 5' end. A single stranded copy of this polynucleotide is then produced to reverse polarity using a primer specific for the primer site in the first domain' (complement of the first domain **102**). The resulting nucleic acid has structure **100** shown in FIG. **1**A, where the first domain **102** includes the

primer site and the MID. Site A (**110**) in FIG. **1** is determined by the specificity of the 5' reflex-B_n primer used.

The reflex process (e.g., as shown in FIG. **1**) is then performed to produce a product in which the primer site and the MID are now in close proximity to the desired site (or region of interest (ROI)) within the original polynucleotide (i.e., the site defined by the primer used, e.g., B_1). The resulting polynucleotide can be used in subsequent analyses as desired by the user (e.g., Roche 454 sequencing technology).

It is noted here that, while not shown in FIGS. **1**A and **1**B, any convenient method for adding adapters to a polynucleotide to be processed as described herein may be used in the practice of the reflex process (adapters containing, e.g., primer sites, polymerase sites, MIDs, restriction enzyme sites, and reflex sequences). For example, adapters can be added at a particular position by ligation. For double stranded polynucleotides, an adapter can be configured to be ligated to a particular restriction enzyme cut site. Where a single stranded polynucleotide is employed, a double stranded adapter construct that possesses an overhang configured to bind to the end of the single-stranded polynucleotide can be used. For example, in the latter case, the end of a single stranded polynucleotide can be modified to include specific nucleotide bases that are complementary to the overhang in the double stranded adaptor using terminal transferase and specific nucleotides. In other embodiments, PCR or linear amplification methods using adapter-conjugated primers is employed to add an adapter at a site of interest. Again, any convenient method for producing a starting polynucleotide may be employed in practicing the methods of the subject invention.

In certain embodiments, the nucleic acid may be sequenced directly using a sequencing primer specific for the primer site. This sequencing reaction will read through the MID and desired site in the insert.

In certain embodiments, the polynucleotide may be isolated (or fractionated) using an appropriate A_n primer (e.g., when using B_1 as the first primer, primer A_1 can be used). In certain embodiments, the A_n primed polynucleotide is subjected to nucleic acid synthesis conditions to produce a copy of the fragment produced in the reflex process. In certain of these embodiments, the A_n primer has appended on its 5' end a primer site that can be used in subsequent steps, including sequencing reactions. Providing a primer site in the A_n primer allows amplifying and/or sequencing from both ends of the resultant fragment: from the primer site in the first domain **102** and the primer site in the A_n primer (not shown in FIG. **1**B). Because of the position of the primer sites and their distance apart (i.e., less than one sequencing run apart), sequencing from both ends will usually capture the sequence of the desired site (or ROI) and the sequence of the MID, which can be used for subsequent bioinformatic analyses, e.g., to positively identify the sample of origin. It is noted here that while sequencing in both directions is possible, it is not necessary, as sequencing from either primer site alone will capture the sequence of the ROI as well as its corresponding MID sequence.

Note that in certain embodiments, the first fragment obtained by amplification/extension from primer B_0 directly, the polarity of the ROI in the resulting fragment is reversed as compared to the ROI in fragments obtained by primers B_1-B_n. This is because the B_0-generated fragment, unlike the B_1-B_n generated fragments, has not been subjected to a reflex process which reverses the orientation of the ROI sequence with respect to the first domain/reflex sequence (as described above). Therefore, the B_0 primer may have appended to it a primer site (e.g., at its 5' end) that can be used for subsequent amplification and/or sequencing reactions (e.g., in Roche 454 sequencing system) rather than a reflex sequence as with primers B_1-B_n. However, in certain embodiments, as noted above, the reflex process may be used with a corresponding B_0-A_0 primer pair as described above, i.e., using a B_0 primer having a 5' reflex sequence and a corresponding A_0 primer with its corresponding 5' adapter domain (e.g., a primer site).

It is noted here that because the particular sections of sequence to be analyzed are defined by the A_n-B_n primer pairs (as shown and described above), a much higher sequence specificity is achieved as compared to using previous extraction methods that employ only a single oligo binding event (e.g., using probes on a microarray).

FIG. **3** provides a detailed flow chart for an exemplary embodiment that employs reflex sequences for use in sequencing multiple specific regions in a polynucleotide (i.e., regions **1**, **2**, **3**, **4** and **5** in an 11 kb region of lambda DNA).

A single parent DNA fragment **202** is generated that includes adapter domains (i.e., a Roche 454 sequencing primer site, a single MID, and a reflex sequence) and the sequence of interest. In the example shown, the sequence of interest is from lambda DNA and the reflex sequence is present on the top strand (with its complement shown in the bottom strand). Any convenient method for producing this parent DNA fragment may be used, including amplification with a primer that includes the adapter domains (e.g., using PCR), cloning the fragment into a vector that includes the adapter domains (e.g., a vector with the adapter domains adjacent to a cloning site), or by attaching adapters to polynucleotide fragments (e.g., fragment made by random fragmentation, by sequence-specific restriction enzyme digestion, or combinations thereof). While only a single fragment with a single MID is shown, the steps in FIG. **3** are applicable to samples having multiple different fragments each with a different MID, e.g., a sample having a population of homologous fragments from any number of different sources (e.g., different individuals). FIG. **3** describes the subsequent enzymatic steps involved in creating the five daughter fragments in which regions **1**, **2**, **3**, **4** and **5** (shown in polynucleotide **204**) are rearranged to be placed within a functional distance of the adapter domains (i.e., close enough to the adapter domains to be sequenced in a single Roche 454 sequencing reaction). Note that certain steps are shown for region **4** only (**206**).

In step **1**, the five regions of interest are defined within the parent fragment (labeled **1** to **5** in polynucleotide **204**) and corresponding primer pairs are designed for each. The distance of each region of interest from the reflex sequence is shown below polynucleotide **204**. The primer pairs are designed as described and shown in FIG. **1**B (i.e., the A_n-B_n primer pairs). For clarity, only primer sites for region **4** are shown in FIG. **3** ("primer sites" surrounding region **4**). In step **2**, sequence specific primer extensions are performed (only region **4** is shown) with corresponding B_n primers to produce single stranded polynucleotides having structure **208** (i.e., having the reflex sequence on the 5' terminus). As shown, the B_n primer for region **4** will include a sequence specific primer site that primes at the 3'-most primer site noted for region **4** (where "3'-most" refers to the template strand, which in FIG. **3** is the top strand). This polynucleotide is copied back to produce polynucleotide **210** having reversed polarity (e.g., copied using a primer that hybridizes to the 454A' domain). Polynucleotide **210** has structure similar to polynucleotide **100** shown at the top of FIG. **1**.

Step **4** depicts the result of the intramolecular priming between the reflex sequence and its complement followed by extension to produce the MID' and 454A' structures at the 3' end (polynucleotide **212**). In the embodiments shown in FIG. **3**, polynucleotide **212** is treated with T7 exonuclease to remove double stranded DNA from the 5' end (as indicated above, this step is optional). The polynucleotide formed for region **4** is shown as **216** with polynucleotides for the other regions also shown (**214**).

It is noted here that the formation of each of the polynucleotides **214** may be accomplished either in separate reactions (i.e., structure with region **1** in proximity to the adapter domains is in a first sample, the structure with region **2** in proximity to the adapter region is in a second sample, etc.) or in one or more combined sample.

In step **6** the polynucleotides **214** are copied to reverse polarity to form polynucleotides **218**. In step **7**, each of these products are then primed with the second primer of the specific primer pair (see A_n primers as shown in FIG. **1**B) each having a second Roche 454 primer site (454B) attached at the 5' end, and extended to form products **220**. Steps **6** and **7** may be combined (e.g., in a single PCR or other amplification reaction).

In summary, FIG. **3** shows how the reflex process can be employed to produce five daughter fragments **220** of similar length (e.g., ~500 bp) each of which contain DNA sequences that differ in their distance from the reflex sequence in the starting structure **202** while maintaining the original MID.

FIG. **4** shows another exemplary use of the reflex process as described herein. In the embodiment shown in FIG. **4**, a target sequence (i.e., containing region of interest "E") is enriched from a pool of adapter-attached fragments. In certain embodiments, the fragments are randomly sheared, selected for a certain size range (e.g., DNA having a length from 100 to 5000 base pairs), and tagged with adapters (e.g., asymmetric adapters, e.g., as described in U.S. patent application Ser. No. 12/432,080, filed on Apr. 29, 2009). The asymmetric adaptor employed in FIG. **4** contains a sequencing primer site (454A, as used in the Roche 454 sequencing platform), an MID, an X sequence, and an internal stem region (ISR), which denotes the region of complementarity for the asymmetric adapter that is adjacent to the adapter attachment site (see, e.g., the description in U.S. application Ser. No. 12/432,080, filed on Apr. 29, 2009, incorporated herein by reference in its entirety). The X sequence can be any sequence that can serve as a binding site for a polynucleotide containing the complement of the X sequence (similar to a primer site). As described below, the X sequence allows for the annealing of an oligonucleotide having a 5' overhang that can serve as a template for extension of the 3' end of the adaptor oligonucleotide. The sequencing direction of the sequencing primer site (454A primer site in structure **401** of FIG. **4**) is oriented such that amplification of the adapter ligated fragment using the sequencing primer site proceeds away from the ligated genomic insert. This has the effect of making the initial asymmetric adapter ligated library 'inert' to amplification using this primer, e.g., in a PCR reaction.

To extract a region of interest (the "E" region), the library is mixed with an oligonucleotide (**403**) containing a 3' X' sequence and a target specific priming sequence (the 1' sequence) under hybridization/annealing conditions. The target specific sequence **1'** is designed to flank one side of the region of interest (the 1' sequence adjacent to E in the genomic insert; note that only the E-containing polynucleotide fragment is shown in FIG. **4**), much like a PCR primer. After annealing primer **403**, the hybridized complex is extended, whereby all of the adaptor tagged fragments will obtain the complement of the target specific sequence (i.e., the 1 sequence) on the 3' end (see structure **405**; arrows denote the direction of extension).

Extended products **405** are then denatured and the 1/1' regions allowed to hybridize intramolecularly in a reflex process priming event, after which nucleic acid extension is performed to form structure **407** (extension is from the 1 priming site; shown with an arrow). This reflex reaction creates a product (**407**) that, unlike its parent structure (**405**), has a sequencing primer site (454A) that is oriented such the extension using this primer sequence proceeds towards the region of interest. Thus, in the absence of a priming and extension reflex reaction, extension with a sequencing primer will not generate a product containing the region of interest (the E region). In other words, only E-region containing target polynucleotides will have a 454A sequence that can amplify genomic material (structure **407**).

After completing the reflex process (using 1/1' as the reflex sequences), a PCR amplification reaction is performed to amplify the region of interest (with associated adapter domains). However, before performing the PCR reaction, the fragment sample is "inactivated" from further extension using terminal transferase and ddNTPs. This inactivation prevents non-target adaptor tagged molecules from performing primer extension from the 3' primer 1 site. Once inactivated, a PCR reaction is performed using a sequencing primer (i.e., 454A primer **409**) and a second primer that primes and extends from the opposite side of the region of interest (i.e., primer **411**, which includes a 5' 454B sequencing primer site and a 3' "2" region that primes on the opposite end of E from the 1 region). Only fragments that have undergone the reflex process and contain the E region will be suitable templates for the PCR reaction and produce the desired product (**413**).

Thus, the process exemplified in FIG. **4** allows for the movement of an adapter domain (e.g., containing functional elements and/or MID) into proximity to a desired region of interest.

The reflex process described herein can be used to perform powerful linkage analysis by combining it with nucleic acid counting methods. Any convenient method for tagging and/or counting individual nucleic acid molecules with unique tags may be employed (see, e.g., U.S. Pat. No. 7,537,897, issued on May 26, 2009; U.S. Pat. No. 7,217,522, issued on May 15, 2007; U.S. patent application Ser. No. 11/377,462, filed on Mar. 16, 2006; and U.S. patent application Ser. No. 12/432,080, filed on Apr. 29, 2009; each of which is incorporated by reference herein in their). All of this can be conducted in parallel thus saving on the cost of labor, time and materials.

In one exemplary embodiment, a large collection of sequences is tagged with MID such that each polynucleotide molecule in the sample has a unique MID. In other words, each polynucleotide in the sample (e.g., each individual double stranded or single stranded polynucleotide) is tagged with a MID that is different from every other MID on every other polynucleotide in the sample. In general, to accomplish such molecular tagging the number of distinct MID tags to be used should be many times greater than the actual number of molecules to be analyzed. This will result in the majority of individual nucleic acid molecules being labeled with a unique ID tag (see, e.g., Brenner et al., Proc. Natl. Acad. Sci. USA. 2000 97(4):1665-70). Any sequences that then result from the reflex process on that particular molecule (e.g., as described above) will thus be labeled with the same unique MID tag and thus inherently linked. Note that

once all molecules in a sample are individually tagged, they can be manipulated and amplified as much as needed for processing so long as the MID tag is maintained in the products generated.

For example, we might want to sequence one thousand viral genomes (or a specific genomic region) or one thousand copies of a gene present in somatic cells. After tagging each polynucleotide in the sample with a sequencing primer site, MID and reflex sequence (as shown in the figures and described above), we use the reflex process to break each polynucleotide into lengths appropriate to the sequencing procedure being used, transferring the sequencing primer site and MID to each fragment (as described above). Obtaining sequence information from all of the reflex-processed samples can be used to determine the sequence of each individual polynucleotide in the starting sample, using the MID sequence to defining linkage relationships between sequences from different regions in the polynucleotide being sequenced. Using a sequencing platform with longer read lengths can minimize the number of primers to be used (and reflex fragments generated).

The advantages noted above are shown in FIG. **5**. This figure shows a comparison of methods for identifying nucleic acid polymorphisms in homologous nucleic acids in a sample (e.g., the same region derived from a chromosomal pair of a diploid cell or viral genomes/transcripts). The top schematic shows two nucleic acid molecules in a sample (1 and 2) having a different assortment of polymorphisms in polymorphic sites A, B and C (A1, B1, C1 and C2). Standard sequencing methods using fragmentation (left side) can identify the polymorphisms in these nucleic acids but do not retain linkage information. Employing the reflex process described herein to identify polymorphisms (right side) maintains linkage information. It is noted that not all domain structures and steps are shown in the reflex process for simplicity.

Kits and Systems

Also provided by the subject invention are kits and systems for practicing the subject methods, as described above, such vectors configured to add reflex sequences to nucleic acid inserts of interest and regents for performing any steps in the cloning or reflex process described herein (e.g., restriction enzymes, nucleotides, polymerases, primers, exonucleases, etc.). The various components of the kits may be present in separate containers or certain compatible components may be precombined into a single container, as desired.

The subject systems and kits may also include one or more other reagents for preparing or processing a nucleic acid sample according to the subject methods. The reagents may include one or more matrices, solvents, sample preparation reagents, buffers, desalting reagents, enzymatic reagents, denaturing reagents, where calibration standards such as positive and negative controls may be provided as well. As such, the kits may include one or more containers such as vials or bottles, with each container containing a separate component for carrying out a sample processing or preparing step and/or for carrying out one or more steps of a nucleic acid variant isolation assay according to the present invention.

In addition to above-mentioned components, the subject kits typically further include instructions for using the components of the kit to practice the subject methods, e.g., to prepare nucleic acid samples for perform the reflex process according to aspects of the subject methods. The instructions for practicing the subject methods are generally recorded on a suitable recording medium. For example, the instructions may be printed on a substrate, such as paper or plastic, etc. As such, the instructions may be present in the kits as a package insert, in the labeling of the container of the kit or components thereof (i.e., associated with the packaging or sub-packaging) etc. In other embodiments, the instructions are present as an electronic storage data file present on a suitable computer readable storage medium, e.g. CD-ROM, diskette, etc. In yet other embodiments, the actual instructions are not present in the kit, but means for obtaining the instructions from a remote source, e.g. via the internet, are provided. An example of this embodiment is a kit that includes a web address where the instructions can be viewed and/or from which the instructions can be downloaded. As with the instructions, this means for obtaining the instructions is recorded on a suitable substrate.

In addition to the subject database, programming and instructions, the kits may also include one or more control samples and reagents, e.g., two or more control samples for use in testing the kit.

Utility

The reflex process described herein provides significant advantages in numerous applications, a few of which are noted below (as well as described above).

For example, as described above, certain aspects of the reflex process define the particular sections of sequence to be analyzed by a primer pair, as in PCR (e.g., the two oligos shown as A_n-B_n in FIG. **1**B). This results in higher sequence specificity as compared to other extraction methods (e.g., using probes on a microarray) that only use a single oligo sequence. The separation of the probes defines a length that can be relatively uniform (hence making subsequent handling including amplification more uniform) and can also be tailored to the particular sequencing platform being employed.

Further, as described above, aspects of the present invention can be used to analyze homologous genomic locations in a multiplexed sample (i.e., a sample having polynucleotides from different genomic samples) in which the polynucleotides are tagged with the MID. This is possible because the reflex process, which operates intramolecularly, maintains the MID thus linking any particular fragment to the sample from which it originates.

Finally, as the reflex processes described herein function intramolecularly, one can determine the genetic linkage between different regions on the same large fragment that are too far apart to be sequenced in one sequence read. Such a determination of linkage may be of great value in plant or animal genetics (e.g., to decide if a particular set of variations are linked together on the same stretch of chromosome) or in viral studies (e.g., to determine if particular variations are linked together on the same stretch of a viral genome/transcripts, e.g., HIV, hepatitis virus, etc.).

EXAMPLES

Example I

FIGS. **6** and **7** provide experimental data and validation of the reflex process described herein using synthetic polynucleotide substrates.

Methods

Substrate:

The 100 base oligonucleotide substrate (as shown diagrammatically in FIG. **6**A) was synthesized with internal fluorescein-dT positioned between the REFLEX and REFLEX' sequences. This label provides convenient and

sensitive method of detection of oligonucleotide species using polyacrylamide gel electrophoresis.

Extension Reactions:

Reactions were prepared which contained 1 μM of the 100 base oligonucleotide substrate, 200 μM dNTPs, presence or absence of 1 μM competitor oligonucleotide, 0.5 μl of each DNA polymerase ("DNAP"): Vent (NEB, 2 units/μl), Taq (Qiagen HotStarTaq 5 units/μl) and Herculase (Stratagene), and made up to 50 μl with the appropriate commercial buffers for each polymerase and dH_2O. For Taq titrations 0.5 μl, 1 μl, 2 μl, and 3 μl enzyme was used in the same 50 μl volume. Reactions were heated in a Biometra thermocycler to 95° C. for 15 minutes (Taq) or 5 minutes (Herculase, Vent), followed by 55° C. or 50° C. for 30 seconds, and a final incubation at 72° C. for 10 minutes.

T7 Exonuclease Digestions:

Reactions were prepared with 10 μl extension reactions above, 0.5 μl T7 exonuclease (NEB, 10 units/μl), and made up to 50 μl using NEB Buffer 4 and dH_2O. Reactions were incubated at 25° C. for 30 minutes.

Gel Electrophoresis Analysis:

An 8% denaturing polyacrylamide gel was used to analyze reaction species. 0.4 μl of extension reactions, and 2 μl of digestion reactions were loaded and ran at 800V for ~1.5 hours. Gels were analyzed for fluorescein using an Amersham/General Electric Typhoon imager.

Results

FIG. **6**A shows the structure of each stage of reflex sequence processing with the expected nucleic acid size shown on the left. The initial single stranded nucleic acid having a sequencing primer site (the Roche 454 sequencing primer A site; listed as 454A); an MID; a reflex sequence; the insert; and a complement of the reflex sequence is 100 nucleotides in length. After self-annealing and extension, the product is expected to be 130 nucleotides in length. After removal of the double stranded region from the 5' end, the nucleic acid is expected to be 82 bases in length.

FIG. **6**B shows the results of three experiments using three different nucleic acid polymerases (Vent, Herculase and Taq, indicated at the top of the lanes). The temperature at which the annealing was carried out is shown at the top of each lane (either 50° C. or 55° C.). The sizes of the three nucleic acids as noted above are indicated on the left and right side of the gel.

As shown in FIG. **6**B, extension appears to be most efficient under the conditions used with Herculase (Herculase is a mixture of two enzymes: modified Pfu DNAP and Archaemax (dUTPase)). Most (or all) of the initial 100 base pair nucleic acid are converted to the 130 base pair product (see lanes 6 and 7). However, after T7 exonuclease digestion the 3'-5' exonuclease activity of Herculase results in partial digestion of the desired 82 base product (note bands at and below the 82 base pairs in lanes 8 and 9).

Taq, which lacks 3'-5' exonuclease activity, shows a stronger band at the expected size of the final product after T7 exonuclease digestion (see lane 13).

FIG. **7** shows the effect on the reflex process of increasing amounts of Taq polymerase as well as the use of a reflex sequence competitor (schematically shown in FIG. **7**A).

As shown in lanes 2 to 5, increased Taq concentration improves extension to ~90% conversion of the starting nucleic acid (see lane 5). Lanes 7 to 8 show that T7 exonuclease digestion does not leave a perfect 82 base product. This may be due to collapse of dsDNA when T7 exonuclease has nearly completed its digestion from the 5' end in the double stranded region of the fold-back structure. It is noted that in many embodiments, the removal of a few additional bases from the 5' end of the polynucleotide will not interfere with subsequent analyses, as nucleotide bases at the 5' end are often removed during subsequent steps.

As shown in Lanes 11-14, addition of a competitor (which can interfere with annealing of the reflex sequences to form the fold-back structure) results in only a small decrease (~5-10%) of fully extended product. Thus, as expected, the intramolecular reaction is heavily favored. Although not shown, we have observed that the competitor oligonucleotide also gets extended by the same amount (~5-10%).

The concentration of the competitor, the concentration of the reflex substrate, and the overall genetic complexity, will all likely affect specific results. The experiments shown in FIGS. **6** and **7** demonstrate that the core parts of the reflex processes as described herein is functional and can be implemented.

Example II

FIG. **8** shows the reflex workflow (diagram at left) and exemplary results of the workflow (gel at right) for a specific region of interest (ROI). The starting material is a double stranded nucleic acid molecule (**700**) that contains a 454A primer site, an MID, a reflex site, and a polynucleotide of interest having three ROIs (2, 3 and 4) at different locations therein. This starting material was subjected to reflex processes (as described in above) specific for ROI 2 as shown in the diagram at the left of the figure, both with and without the use of a T7 exonuclease step (the T7 exonuclease step is shown in the diagram is indicated as "Optional").

Completion of all steps shown in the reflex process should result in a double stranded polynucleotide of 488 base pairs (**702**) with or without the T7 exonuclease step.

As shown in the gel on the right of FIG. **8**, the 488 base pair product was produced in reflex processes with and without the T7 exonuclease step.

FIG. **9** shows an exemplary protocol for a reflex process based on the results discussed above. The diagram shows specific reflex process steps with indications on the right as to where purification of reaction products is employed (e.g., using Agencourt SPRI beads to remove primer oligos). One reason for performing such purification steps is to reduce the potential for generating side products in a reaction (e.g., undesirable amplicons). While FIG. **9** indicates three purification steps, fewer or additional purification steps may be employed depending on the desires of the user. It is noted that the steps of reversing polarity, reflex priming and extension, and "stretch out" (or denaturation)/second reversing polarity step can be performed without intervening purification steps.

The protocol shown in FIG. **9** includes the following steps:

annealing a first primer containing a 5' reflex sequence (or reflex tail, as noted in the figure) specific for the 3' primer site for the R' region to the starting polynucleotide and extending (the primer anneals to the top strand at the primer site at the right of R in polynucleotide **902**, indicated with a *; this step represents the first denature, anneal and extend process indicated on the right);

after purification, adding a 454A primer and performing three cycles of denaturing, annealing and extending: the first cycle results in the copy-back from the 454A primer to reverse the polarity of the strand just synthesized; the second cycle breaks apart the double stranded structure produced, allows the reflex structure to form and then extend; the third cycle results in another copy-back using the same 454A primer originally added;

after purification, adding a second primer specific for the second primer site for the R' region having a 5' 454B tail (this primer anneals to the primer site **3'** of the R' region in polynucleotide **904**, indicated with a *) and denaturing, annealing and extending resulting in a polynucleotide product having 454A and 454B sites surrounding the MID, the reflex sequence, and R'. Note that the first primer specific for the R' region and the second primer specific for the R' region define its boundaries, as described above and depicted in FIG. **1**B);

after another purification, adding 454A and 454B primers and performing a PCR amplification reaction.

Example III

As described above, a reflex sequence can be an "artificial" sequence added to a polynucleotide as part of an adapter or can be based on a sequence present in the polynucleotide of interest being analyzed, e.g., a genomic sequence (or "non-artificial").

The data shown in prior Examples used "artificial" reflex sites. In this Example, the reflex site is a genomic sequence present in the polynucleotide being analyzed.

The starting material is a double stranded DNA containing a 454A site, an MID and a polynucleotide to be analyzed. The 454A and MID were added by adapter ligation to parent polynucleotide fragments followed by enrichment of the polynucleotide to be analyzed by a hybridization-based pull-out reaction and subsequent secondary PCR amplification (see Route **1** in FIG. **13**). Thus, the reflex site employed in this example is a sequence normally present at the 5' end of the subject polynucleotide (a genomic sequence). The polynucleotide being analyzed includes a region of interest distal to the 454A and MID sequences that is 354 base pairs in length.

This starting double stranded nucleic acid is 755 base pairs in length. Based on the length of each of the relevant domains in this starting nucleic acid, the reflex process should result in a product of 461 base pairs.

FIG. **10** shows the starting material for the reflex process (left panel) and the resultant product generated using the reflex process (right panel; reflex process was performed as described in Example II, without using a T7 exonuclease step). A size ladder is included in the left hand lane of each gel to allow estimation of the size of the test material. This figure shows that the 755 base pair starting nucleic acid was processed to the expected 461 base pair product, thus confirming that a "non-artificial" reflex site is effective in moving an adapter domain from one location to another in a polynucleotide of interest in a sequence specific manner.

Example IV

FIG. **11** shows a schematic of an experiment in which the reflex process is performed on a single large initial template (a "parent" fragment) to generate 5 different products ("daughter" products) each having a different region of interest (i.e., daughter products are produced having either region **1**, **2**, **3**, **4** or **5**). The schematic in FIG. **11** shows the starting fragment (11,060 base pairs) and resulting products (each 488 base pairs) generated from each of the different region of interest-specific reflex reactions (reflex reactions are performed as described above). The panel (gel) on the bottom of FIG. **11** shows the larger starting fragment (Lane 1) and the resulting daughter products for each region-specific reflex reaction (lanes 2 to 6, with the region of interest noted in each in the box), where the starting and daughter fragments have the expected lengths. Sequencing of the products confirmed the identity of the region of interest in each of the reflex products shown in the gel. These results demonstrate that multiple different reflex products can be generated from a single, asymmetrically tagged parent fragment while maintaining the adapter domains (e.g., the primer sites and MID).

Example V

FIG. **12** details experiments performed to determine the prevalence of intramolecular rearrangement (as desired in the reflex process) vs. intermolecular rearrangement. Intermolecular rearrangement is undesirable because it can lead to the transfer of an MID from one fragment to another (also called MID switching). MID switching can occur if a reflex sequence in a first fragment hybridizes to its complement in a second fragment during the reflex process, leading to appending the MID from the second fragment to the first fragment. Thus, intermolecular rearrangement, or MID switching, should be minimized to prevent the transfer of an MID from one fragment in the sample to another, which could lead to a misrepresentation of the source of a fragment.

To measure the prevalence of MID switching under different reflex conditions, fragments having different sizes were generated that included two different MIDs, as shown in the top panel of FIG. **12**. The common sequence on these fragments serves as the priming site for the first extension reaction to add the second reflex sequence (see, e.g., step **2** of FIG. **3**). Three exemplary fragments are shown in FIG. **12** for each different fragment size (i.e., 800 base pairs with an MIDB and MIDA combination; 1900 base pairs with MIDC and MIDA combination; and 3000 base pairs with MIDD and MIDA combination). For each MID family (A, B, C and D), there are 10 different members (i.e., MIDA had 10 different members, MIDB has 10 different members, etc.). A set of 10 dual MID fragments for each different size fragment (i.e., 800, 1900 and 3000 base pairs) were generated, where the MID pairs (i.e., MIDA/MIDB, MIDA/MIDC, and MIDA/MIDD) were designated as 1/1, 2/2, 3/3, 4/4, 5/5, 6/6, 7/7, 8/8, 9/9, and 10/10. All 10 fragments of the same size were then mixed together and a reflex protocol was performed.

Due to the domain structure of the fragments, a successful reflex process results in the two MIDs for each fragment being moved to within close enough proximity to be sequenced in a single read using the Roche 454 sequencing platform (see the reflex products shown in the schematic of FIG. **12**). The reflex reactions for each fragment size were performed at four different fragment concentrations to determine the effect of this parameter, as well as fragment length, in the prevalence of MID switching. The reflex products from each reaction performed were subjected to 454 sequencing to determine the identity of both MIDs on each fragment, and thereby the proportion of MID switching that occurred.

The panel on the bottom left of FIG. **12** shows the rate of MID switching (Y axis, shown in % incorrect (or switched) MID pair) for each different length fragment at each different concentration (X axis; 300, 30, 3 and 0.3 nM). As shown in this panel, the MID switch rate decreases with lower concentrations, as would be expected, because intermolecular, as opposed to intramolecular, binding events are concentration dependent (i.e., lower concentrations lead to reduced intermolecular hybridization/binding). In addition, the MID switch rate decreases slightly with length. This is

somewhat unexpected as the ends of longer DNA fragments are effectively at a lower concentration with respect to one another. The reasons for why we do not see this is probably because the production of reflex priming intermediates continues during the final PCR, which means that reflex priming reactions are happening continuously which contributes to MID switching. It is probably the case that the shorter reflex products are able to undergo a higher rate of 'background' reflexing, and therefore increase the overall MID switch rate a little.

These results demonstrate that MID switching can be minimized (e.g., to below 2%, below 1% or even to nearly undetectable levels) by altering certain parameters of the reaction, e.g., by reducing fragment concentration and/or fragment length.

The panel on the bottom right of FIG. **12** shows the frequency of MID switching in the reflex process for the 800 base pair fragments (i.e., MIDA/MIDB containing fragments). In this figure, the area of each circle is proportional to the number of reads containing the corresponding MIDA and MIDB species (e.g., MIDA1/MIDB1; MIDA1/MIDB2; etc.). Thus, a circle representing 200 reads will be 40 times larger in terms of area than a circle representing 5 reads.

As noted above, the MIDA/MIDB combinations having the same number (shown on the X and Y axis, respectively) represent the MIDA/MIDB combinations present in the sample prior to the reflex process being performed (i.e., MIDA/MIDB combinations 1/1, 2/2, 3/3, 4/4, 5/5, 6/6, 7/7, 8/8, 9/9, and 10/10 were present in the starting sample). All other MIDA/MIDB combinations identified by Roche 454 sequencing were the result of MID switching.

This figure shows that the MID switching that occurs during the reflex process is random, i.e., that MID switching is not skewed based on the identity of the MIDB in the reaction).

Exemplary Reflex Protocols

FIG. **13** shows a diagram of exemplary protocols for performing the reflex process on pools of nucleic acids, for example, pools of nucleic acids from different individuals, each of which are labeled with a unique MID. In Route **3**, a pooled and tagged extended library is subjected directly to a reflex process. In Route **2**, the pooled library is enriched by target-specific hybridization followed by performing the reflex process. In Route **1** employs enrichment by PCR amplification. As shown in FIG. **13**, PCR enrichment can be performed directly on the pooled tagged extended library or in a secondary PCR reaction after a hybridization-based enrichment step has been performed (as in Route **2**) to generate an amplicon substrate that is suitable for the reflex process. Additional routes for preparing a polynucleotide sample for performing a reflex process can be implemented (e.g., having additional amplification, purification, and/or enrichment steps), which will generally be dependent on the desires of the user.

Although the foregoing invention has been described in some detail by way of illustration and example for purposes of clarity of understanding, it is readily apparent to those of ordinary skill in the art in light of the teachings of this invention that certain changes and modifications may be made thereto without departing from the spirit or scope of the appended claims.

Accordingly, the preceding merely illustrates the principles of the invention. It will be appreciated that those skilled in the art will be able to devise various arrangements which, although not explicitly described or shown herein, embody the principles of the invention and are included within its spirit and scope. Furthermore, all examples and conditional language recited herein are principally intended to aid the reader in understanding the principles of the invention and the concepts contributed by the inventors to furthering the art, and are to be construed as being without limitation to such specifically recited examples and conditions. Moreover, all statements herein reciting principles, aspects, and embodiments of the invention as well as specific examples thereof, are intended to encompass both structural and functional equivalents thereof. Additionally, it is intended that such equivalents include both currently known equivalents and equivalents developed in the future, i.e., any elements developed that perform the same function, regardless of structure. The scope of the present invention, therefore, is not intended to be limited to the exemplary embodiments shown and described herein. Rather, the scope and spirit of present invention is embodied by the appended claims.

What is claimed:

1. A composition for multiplexed nucleic acid analysis, comprising:

a plurality of beads, wherein the beads are covalently attached to a plurality of oligonucleotide tags, and wherein an oligonucleotide tag of said plurality of oligonucleotide tags comprises:

(a) a first tag sequence configured to distinguish a sample polynucleotide originating from a cell from sample polynucleotides originating from other cells; and

(b) a second tag sequence configured to distinguish said sample polynucleotide from other sample polynucleotide from the same cell and having the same sequence as said sample polynucleotide.

2. The composition of claim **1**, wherein an oligonucleotide tag of said plurality of oligonucleotide tags comprises a sequencing adaptor.

3. The composition of claim **1**, wherein an oligonucleotide tag of said plurality of oligonucleotide tags comprises a capture sequence that is configured to capture said sample polynucleotides.

4. The composition of claim **3**, wherein said capture sequence is a poly-dT sequence.

5. The composition of claim **1**, wherein said plurality of oligonucleotide tags are generated by combinatorial synthesis from a defined set of subunits.

6. The composition of claim **1**, wherein the first tag sequence has a length of 8 to 20 nucleotides.

7. The composition of claim **1**, wherein the second tag sequence has a length of 8 to 20 nucleotides.

8. The composition of claim **1**, wherein said plurality of oligonucleotide tags comprises 1,000 different first tag sequences.

9. The composition of claim **8**, wherein said plurality of oligonucleotide tags comprises 10,000 different first tag sequences.

10. The composition of claim **9**, wherein said plurality of oligonucleotide tags comprises 1,000,000 different first tag sequences.

11. The composition of claim **1**, wherein said plurality of oligonucleotide tags comprises 1,000 different second tag sequences.

12. The composition of claim **11**, wherein said plurality of oligonucleotide tags comprises 10,000 different second tag sequences.

13. The composition of claim **12**, wherein said plurality of oligonucleotide tags comprises 1,000,000 different second tag sequences.

* * * * *

Exhibit F

US010400280B2

(12) **United States Patent**
Hindson et al.

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(54) **METHODS AND SYSTEMS FOR PROCESSING POLYNUCLEOTIDES**

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This patent is subject to a terminal disclaimer.

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(60) Continuation of application No. 16/212,441, filed on Dec. 6, 2018, which is a continuation of application No. 16/052,431, filed on Aug. 1, 2018, now Pat. No. 10,273,541, which is a continuation-in-part of application No. 16/000,803, filed on Jun. 5, 2018, which is a continuation of application No. 15/850,241, filed on Dec. 21, 2017, which is a continuation of application No. 15/588,519, filed on May 5, 2017, now Pat. No. 9,856,530, which is a continuation of application No. 15/376,582, filed on Dec. 12, 2016, now Pat. No. 9,701,998, which is a continuation-in-part of application No. 14/104,650, filed on Dec. 12, 2013, now Pat. No. 9,567,631, and a continuation-in-part of application No. 14/250,701, filed on Apr. 11, 2014, now abandoned, which is a continuation of application No. 14/175,973, filed on Feb. 7, 2014, now Pat. No. 9,388,465, application No. 16/231,185, filed on Dec. 21, 2018, which is a continuation-in-part of application No. 15/847,752, filed on Dec. 19, 2017, which is a continuation of application No. 15/717,871, filed on Sep. 27, 2017, now Pat. No. 9,951,386, which is a continuation-in-part of application No. 14/752,641, filed on Jun. 26, 2015, said application No. 16/052,431 is a continuation-in-part of application No. 15/598,898, filed on May 18, 2017, which is a continuation of application No. 14/624,468, filed on Feb. 17, 2015, now Pat. No. 9,689,024, which is a division of application No. 13/966,150, filed on Aug. 13, 2013, now abandoned, application No. 16/231,185, filed on Dec. 21, 2018, which is a continuation-in-part of application No. 16/052,486, filed on Aug. 1, 2018, which is a continuation-in-part of application No. 16/000,803, and a continuation-in-part of application No. 14/316,447, filed on Jun. 26, 2014, now Pat. No. 10,221,442, which is a continuation-in-part of application No. 13/966,150, and a continuation-in-part of application No. PCT/US2013/054797, filed on Aug. 13, 2013.

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(58) **Field of Classification Search**

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See application file for complete search history.

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(57) **ABSTRACT**

The present disclosure provides compositions, methods, systems, and devices for polynucleotide processing. Such polynucleotide processing may be useful for a variety of applications, including polynucleotide sequencing.

46 Claims, 22 Drawing Sheets

Related U.S. Application Data

application No. 61/800,223, filed on Mar. 15, 2013, provisional application No. 61/762,435, filed on Feb. 8, 2013, provisional application No. 62/061,567, filed on Oct. 8, 2014, provisional application No. 62/017,558, filed on Jun. 26, 2014, provisional application No. 61/683,192, filed on Aug. 14, 2012, provisional application No. 61/896,060, filed on Oct. 26, 2013, provisional application No. 61/909,974, filed on Nov. 27, 2013, provisional application No. 61/991,018, filed on May 9, 2014, provisional application No. 61/940,318, filed on Feb. 14, 2014, provisional application No. 61/937,344, filed on Feb. 7, 2014.

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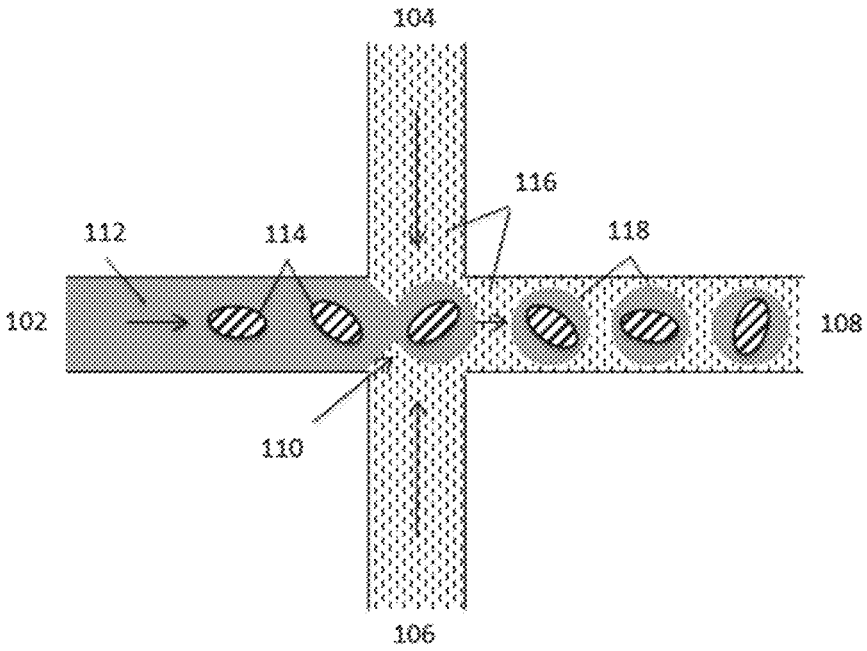

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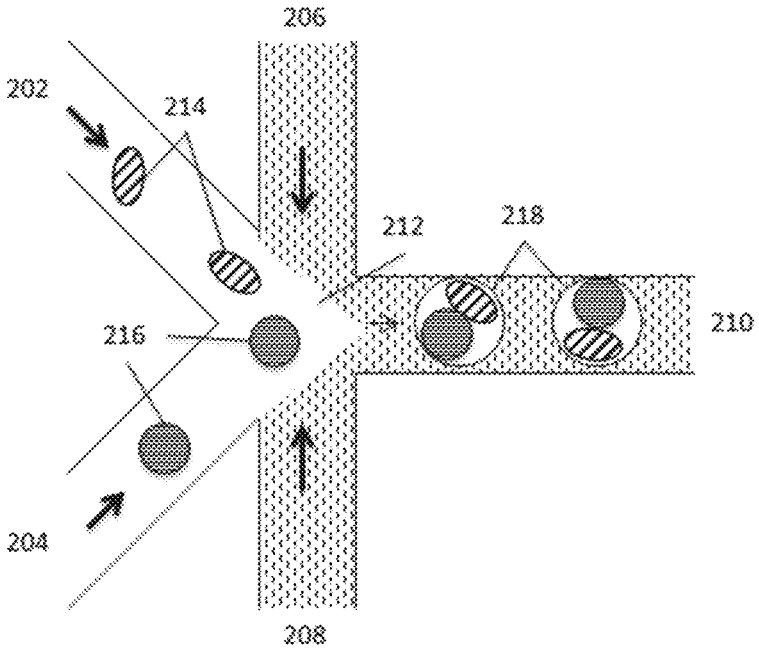

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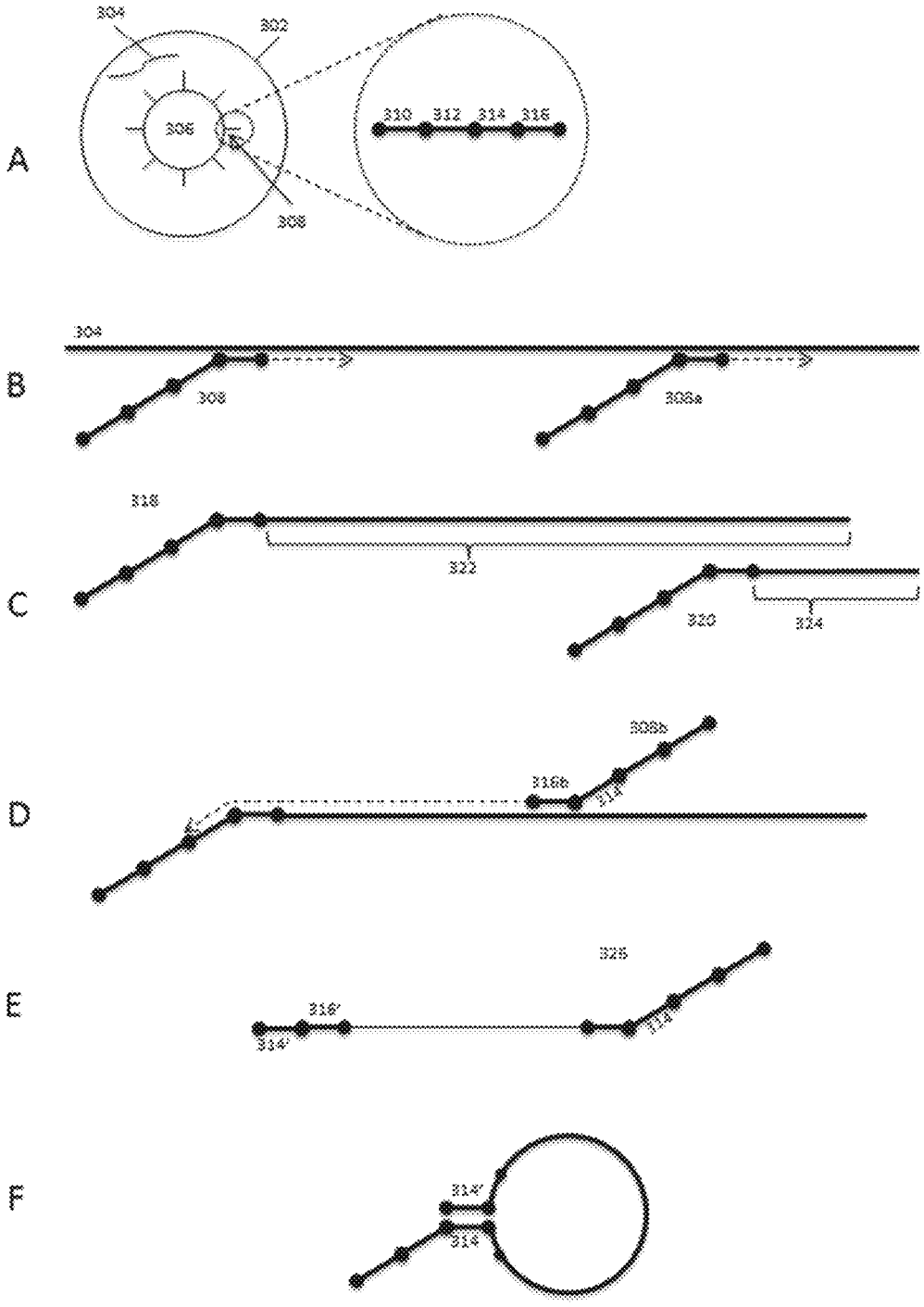
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Figure 3

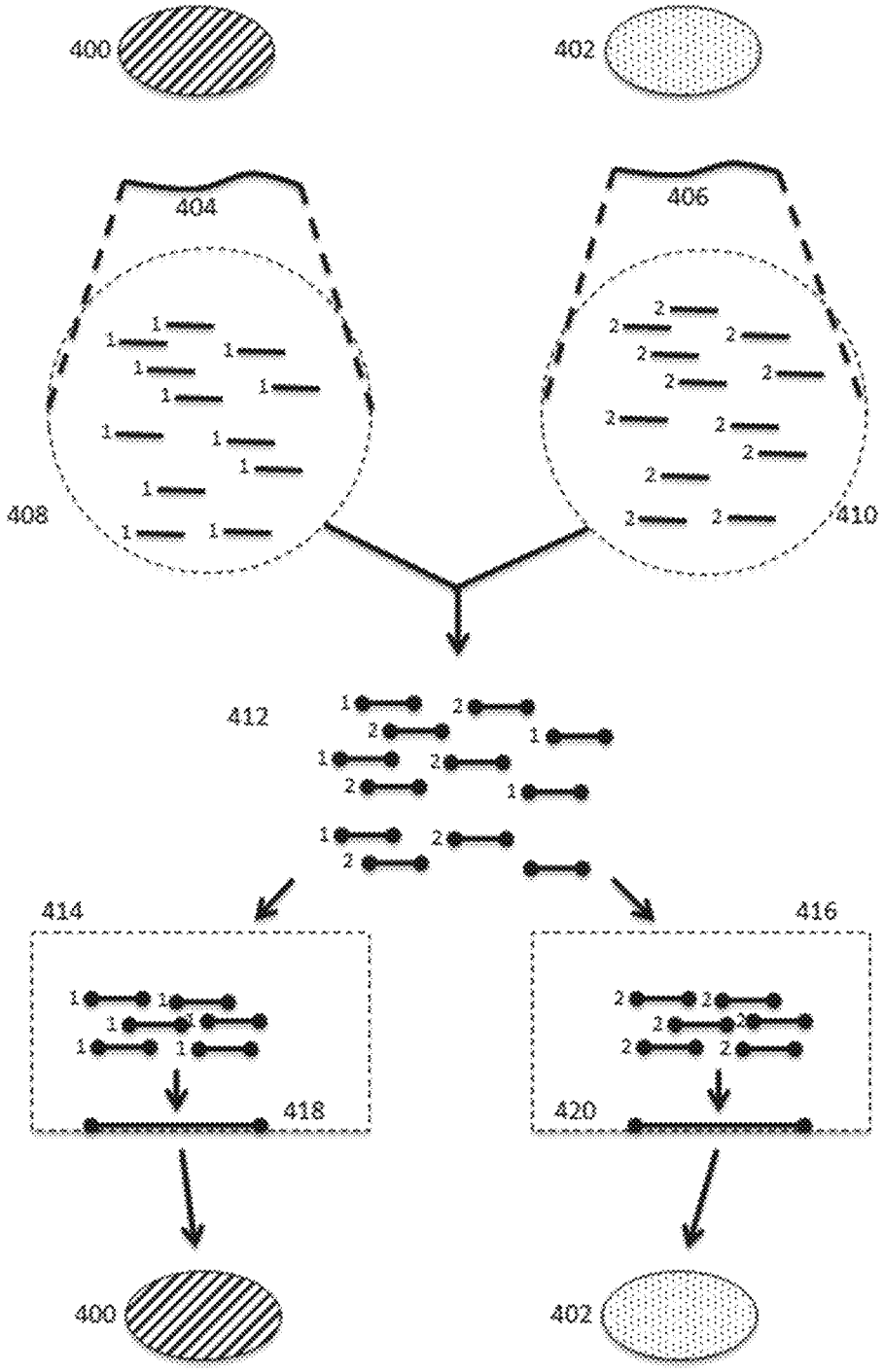


Figure 4

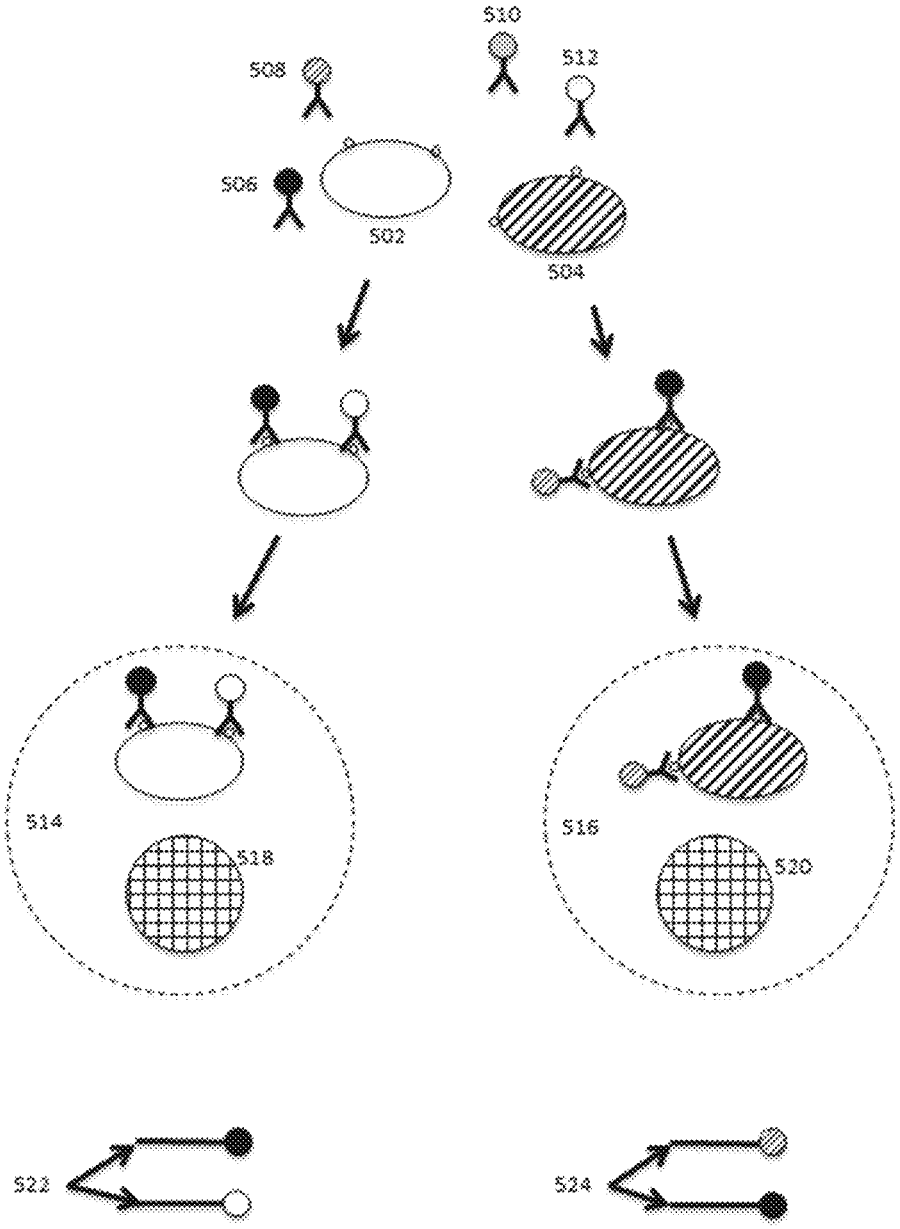
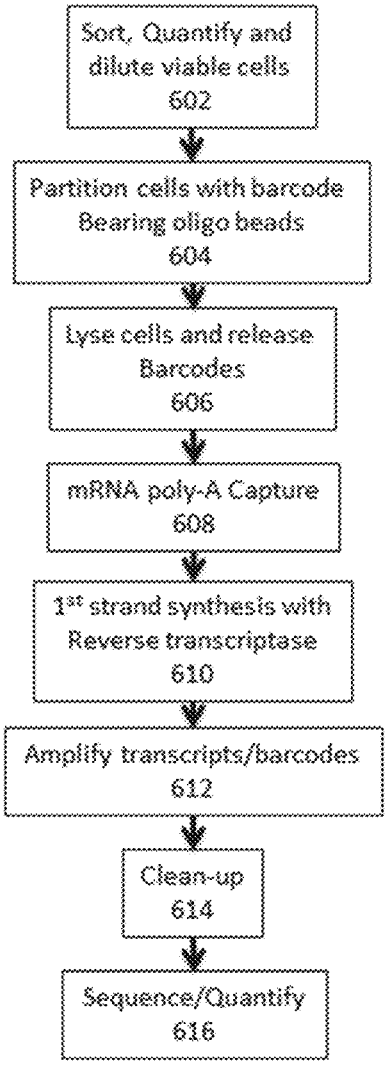


Figure 5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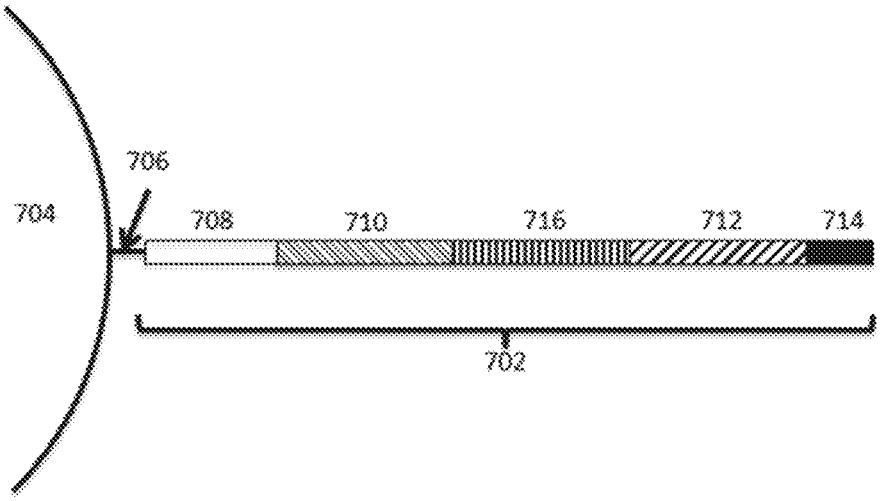
Amplify transcripts/barcodes 612
Clean-up 614
Sequence/Quantify 616

Figure 6



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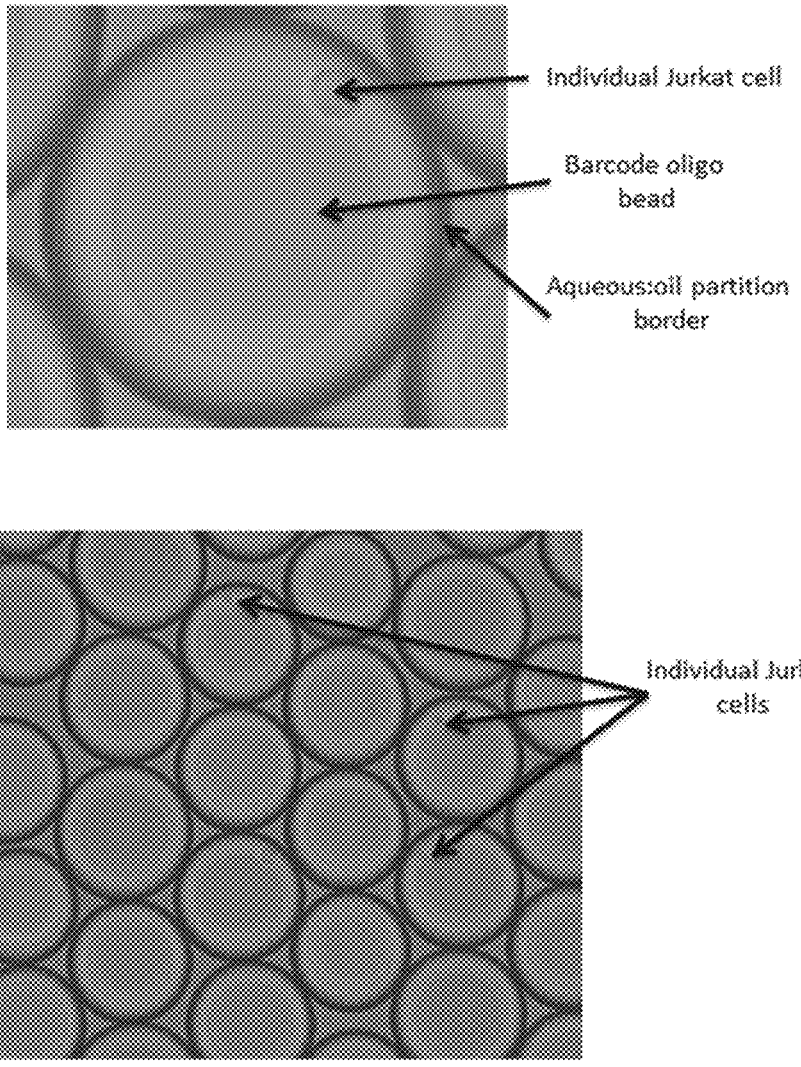
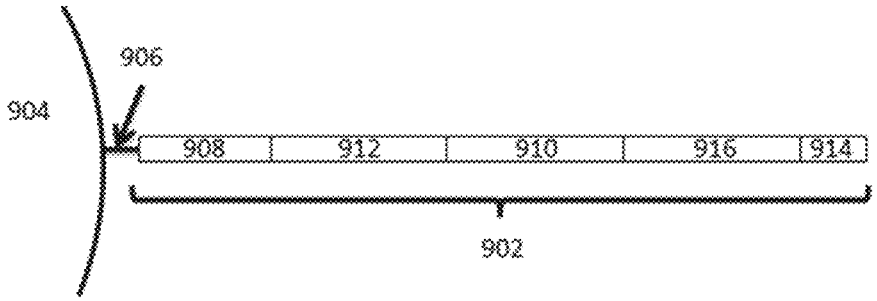

Individual Jurkat cell
Barcode oligo bead
Aqueous:oil partition border
Individual Jurkat cells

Figure 8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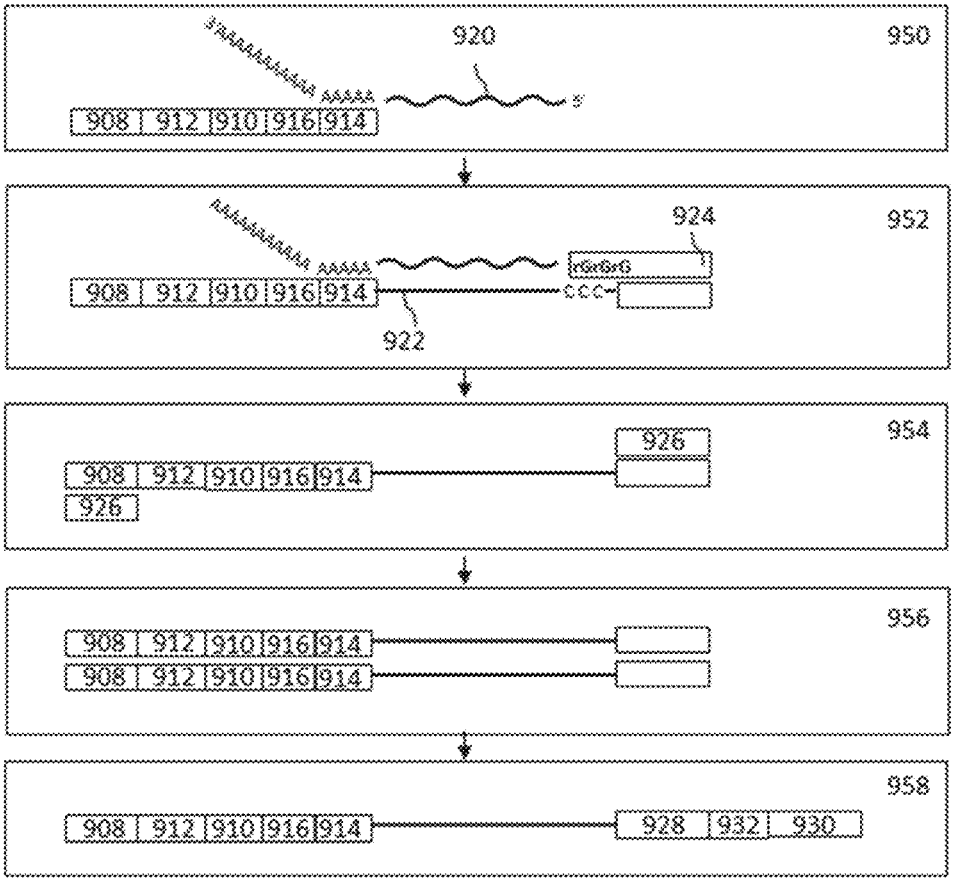
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Figure 9A

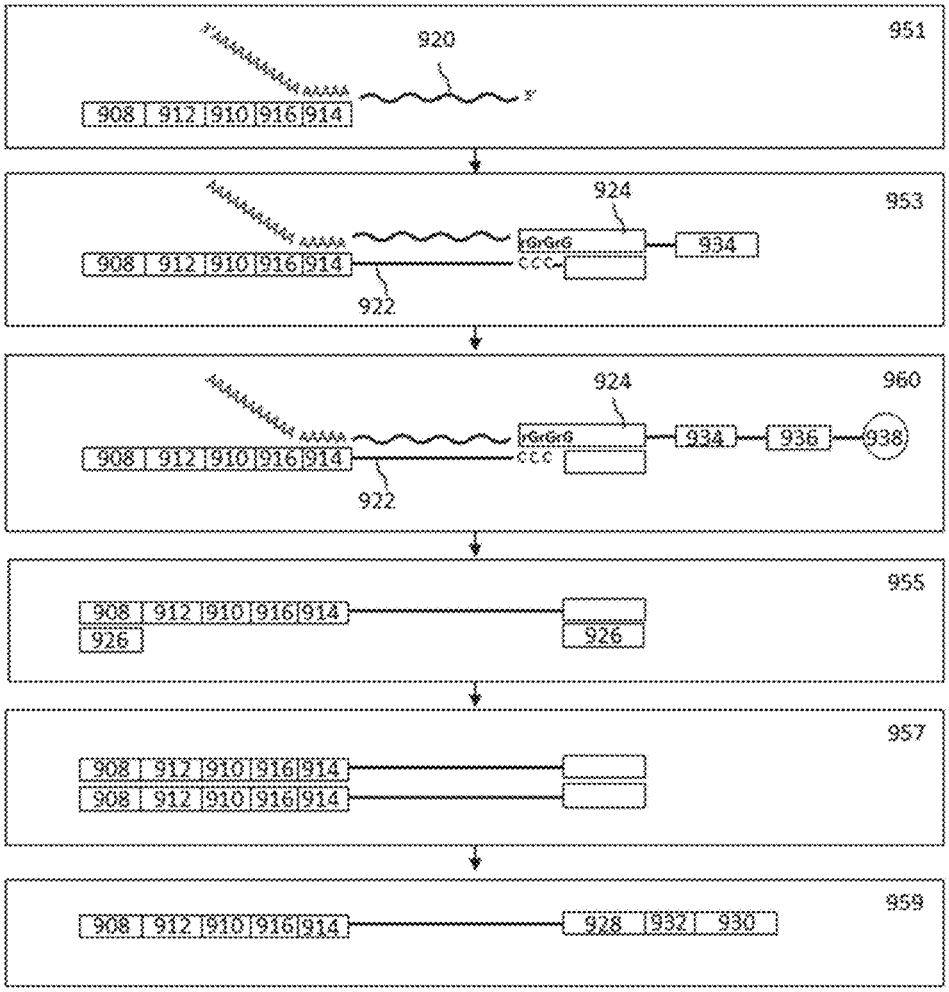
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Figure 9B

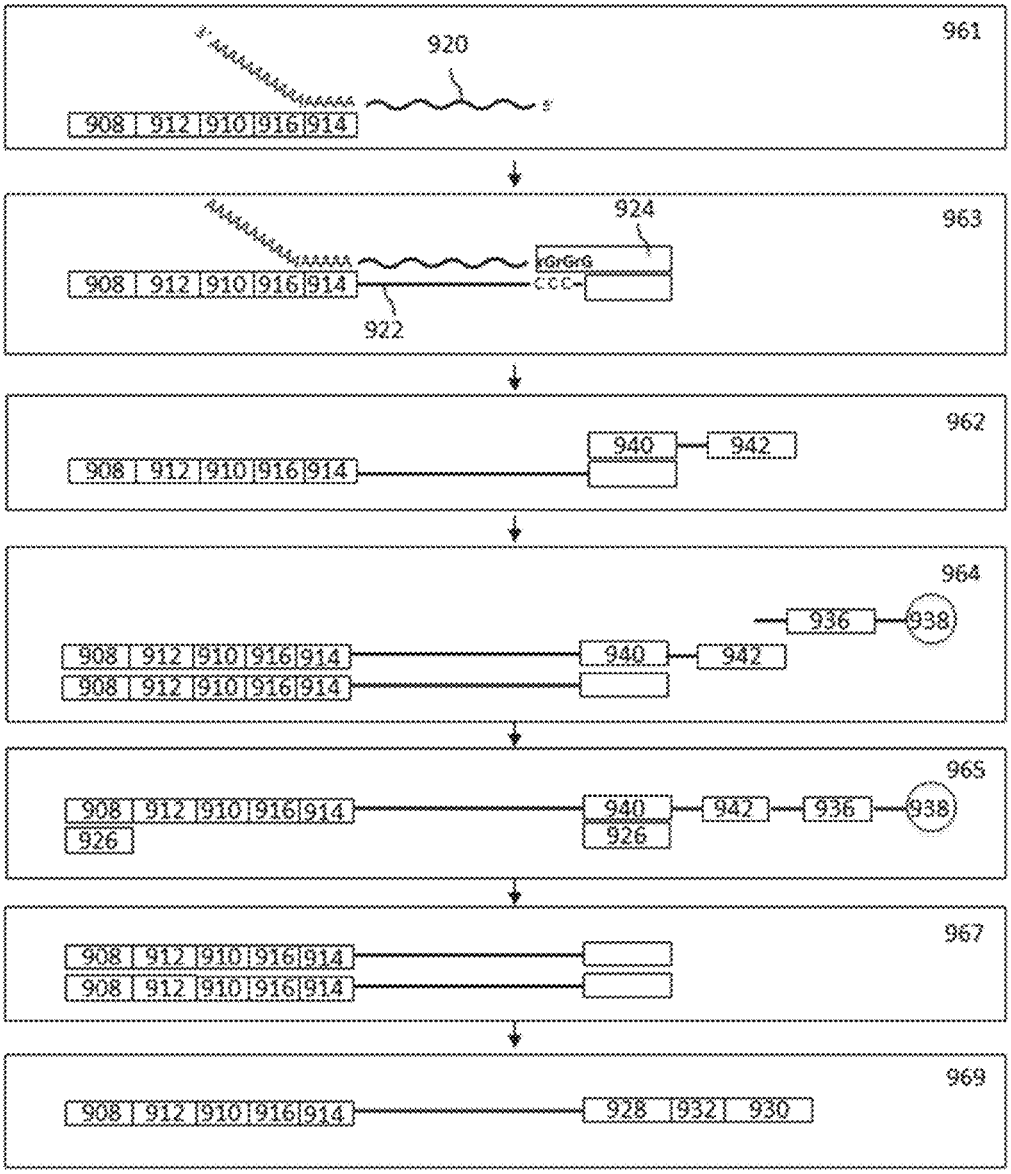
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Figure 9C

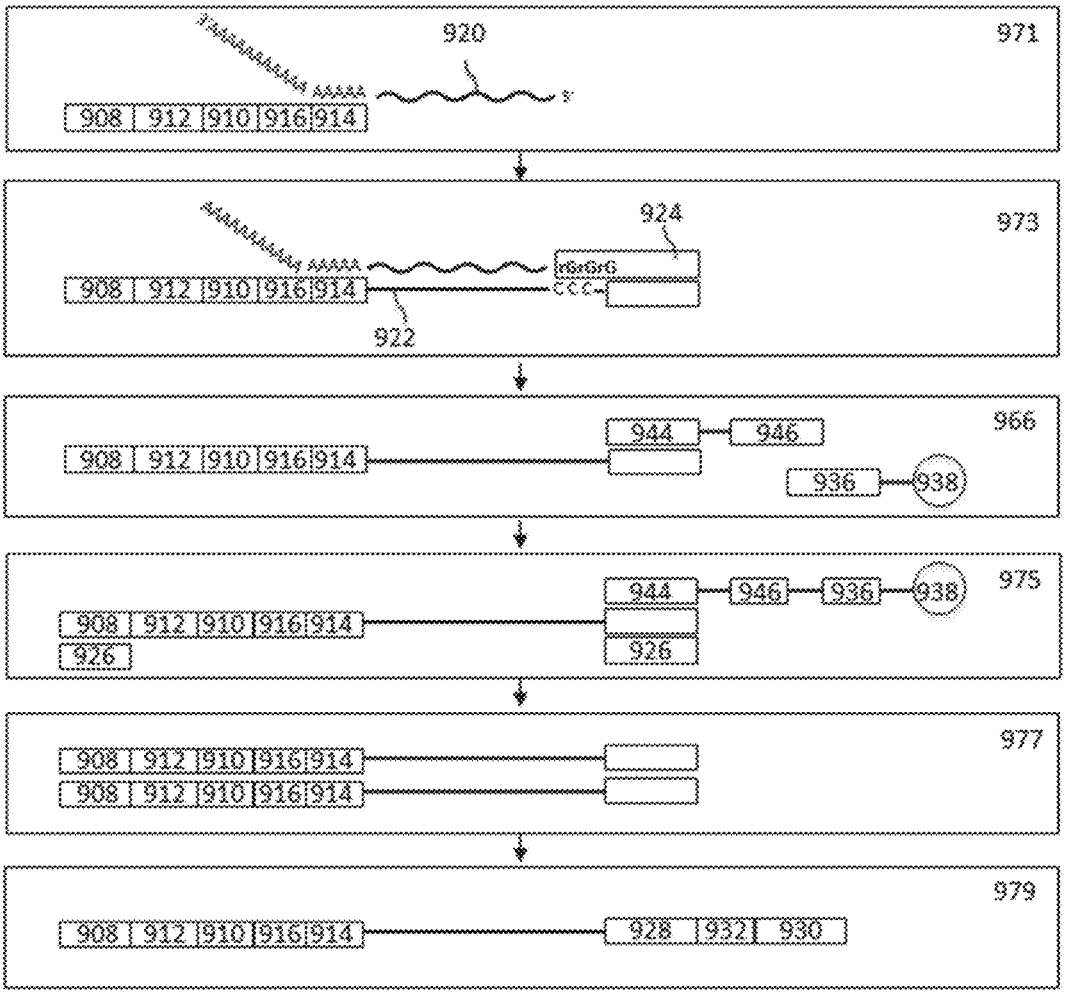
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Figure 9D

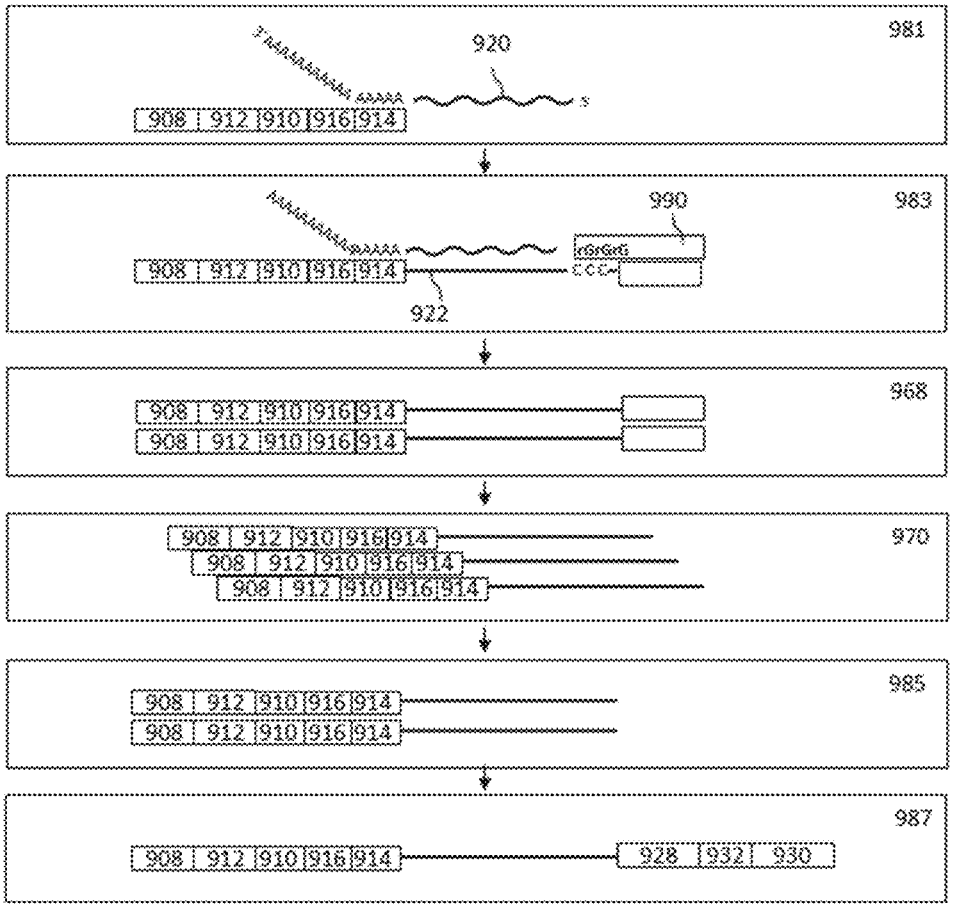
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Figure 9E

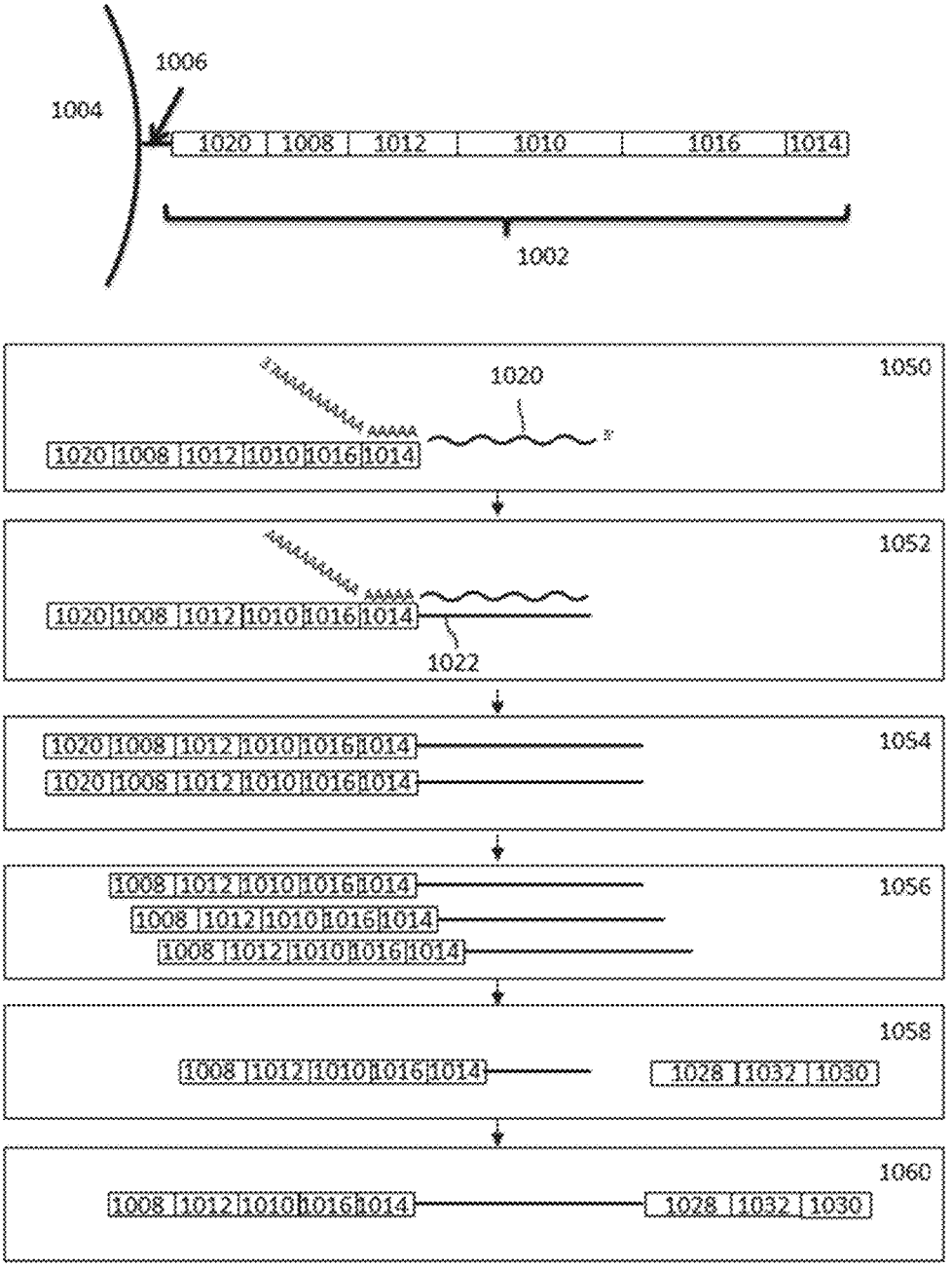
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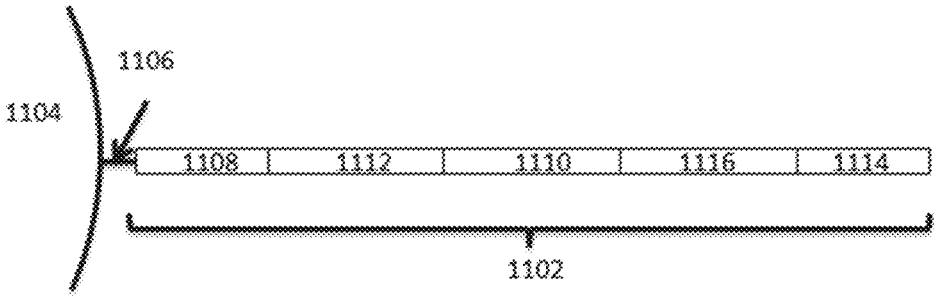
Figure 10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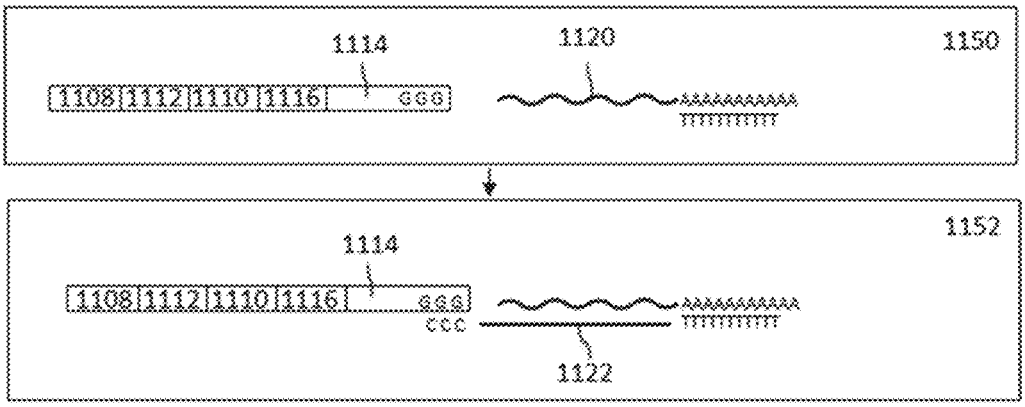
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Figure 11

Figure 12A

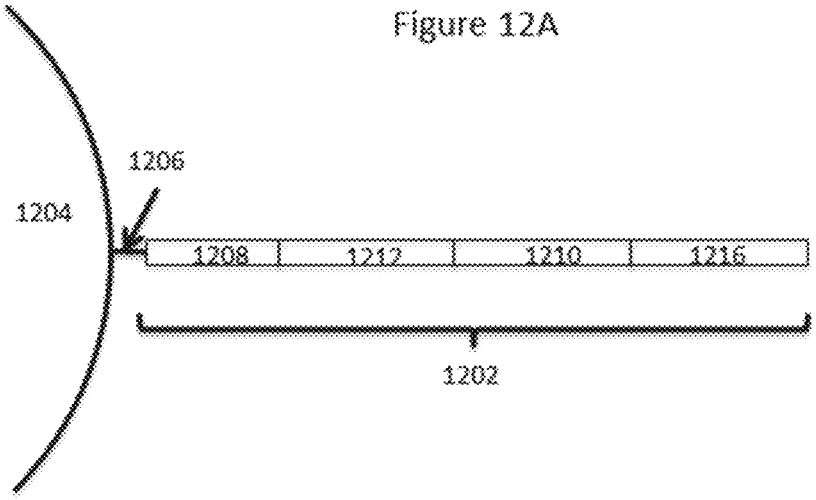


Figure 12B

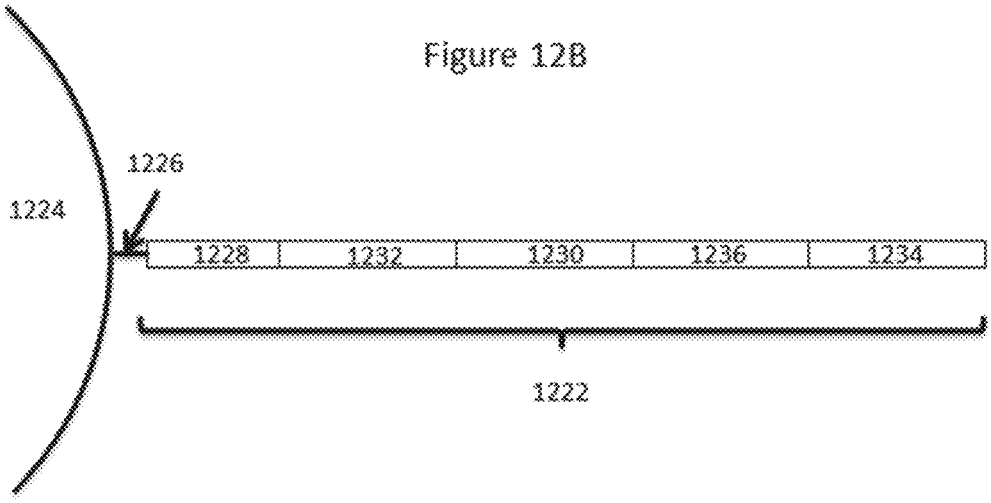


Figure 13A

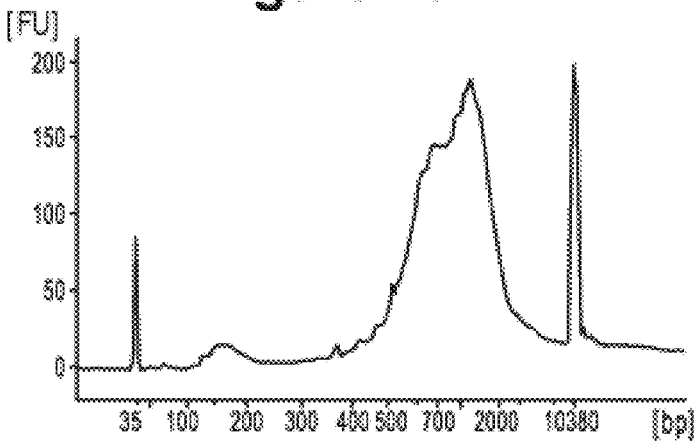


Figure 13B

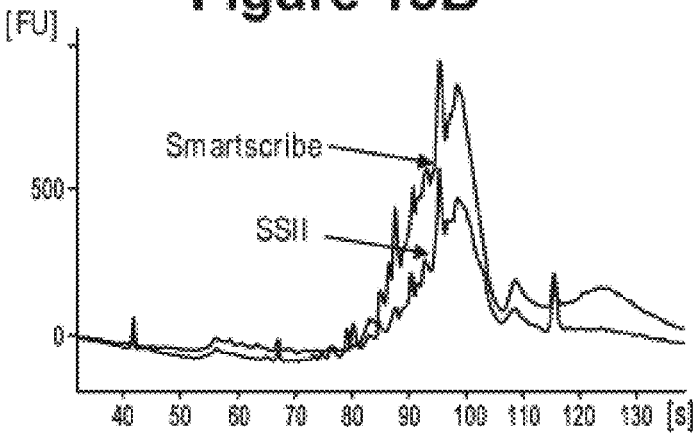


Figure 13C

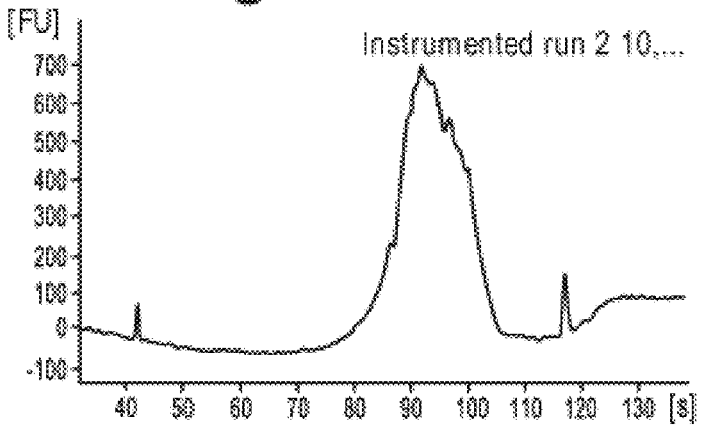


Figure 14A

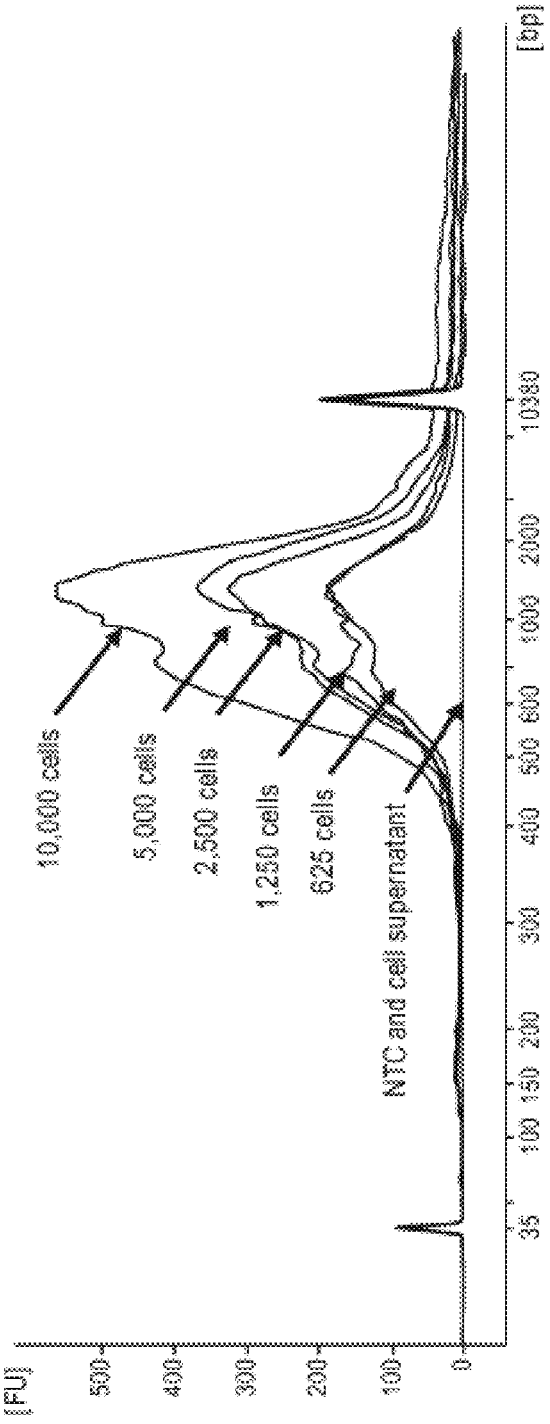
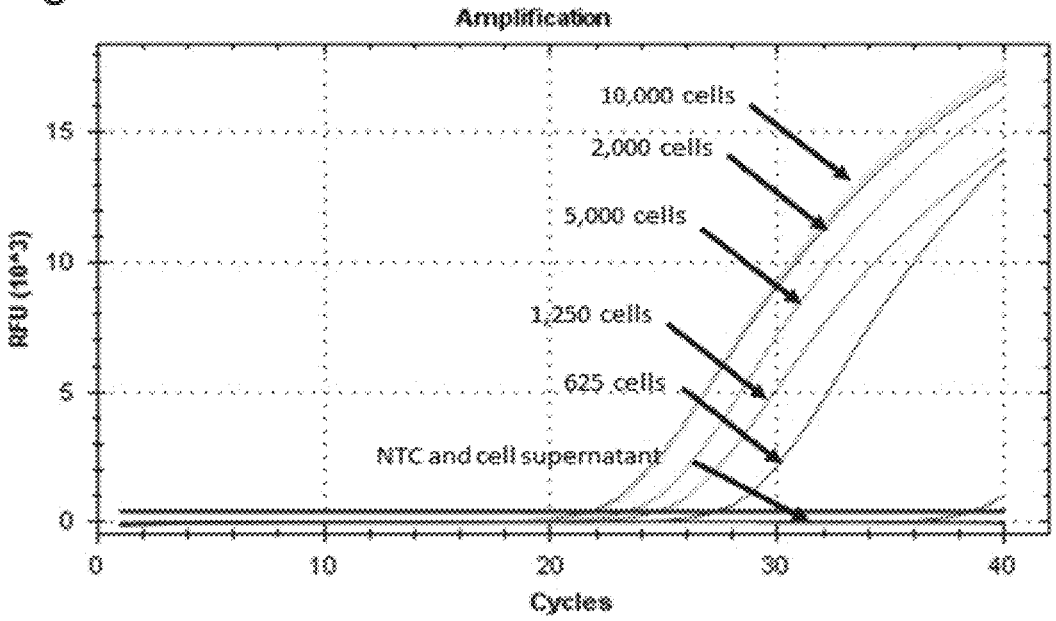

[FU]
500
400
300
200
100
0
35
100
150
200
300
400
500
600
1000
2000
10380
[bp]
10,000 cells
5,000 cells
2,500 cells
1,250 cells
625 cells
NTC and cell supernatant

Figure 14B


Amplification
10,000 cells
2,000 cells
5,000 cells
1,250 cells
625 cells
NTC and cell supernatant
RFU (10^3)
15
10
5
0
0
10
20
30
40
Cycles

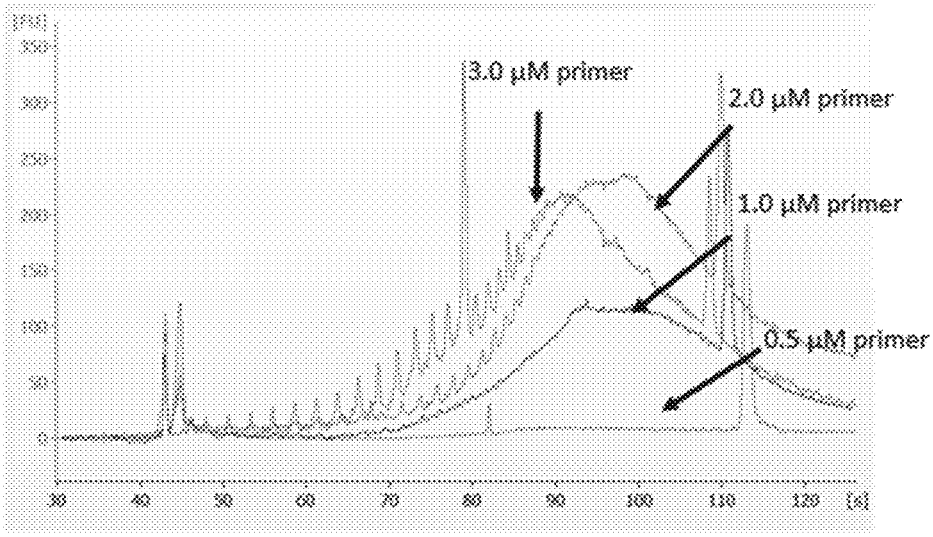


Figure 15

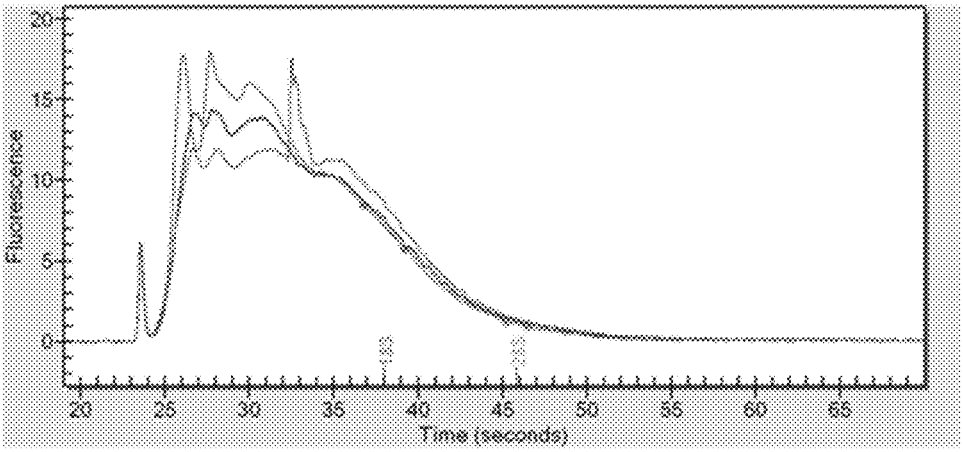

20
15
10
5
0
Fluorescence
20
25
30
35
40
45
50
55
60
65
Time (seconds)

Figure 16

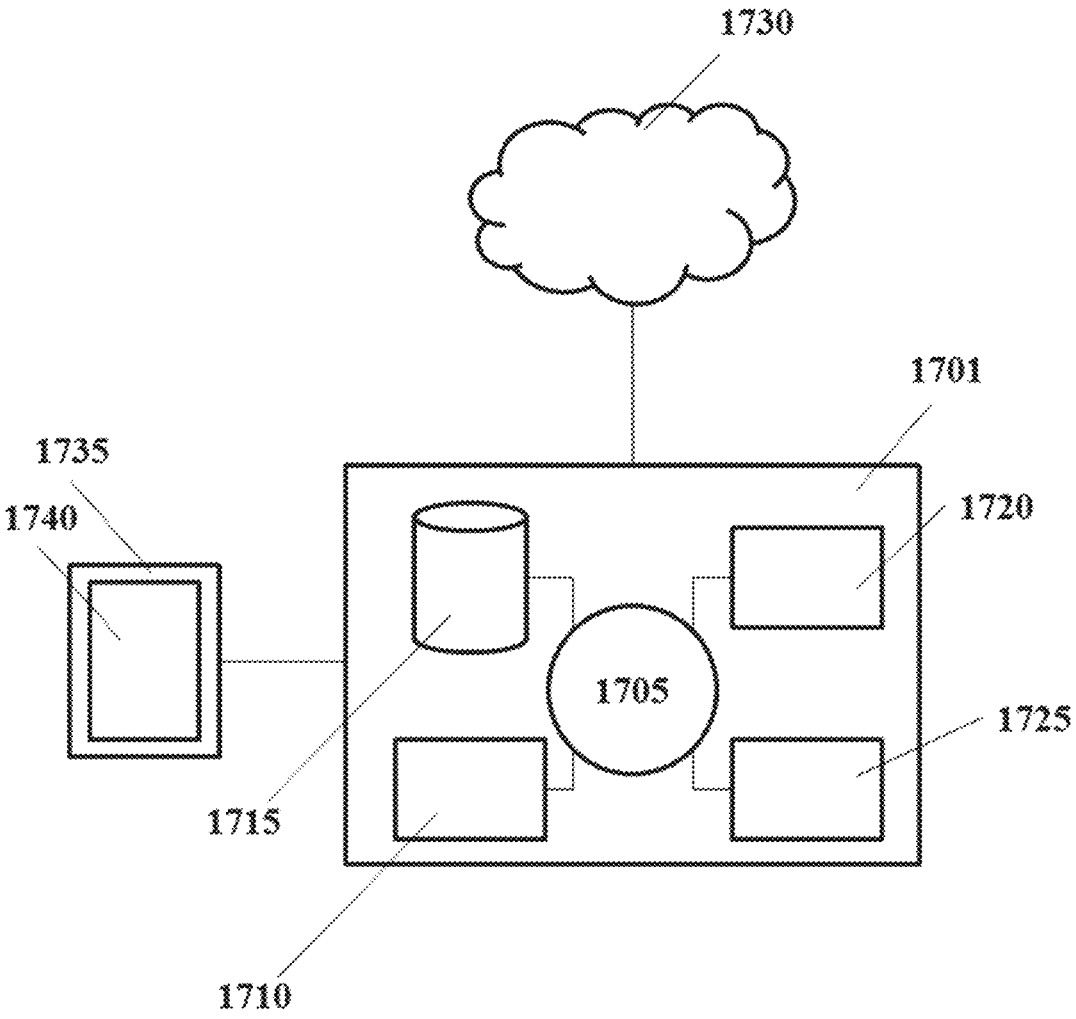


Figure 17

METHODS AND SYSTEMS FOR PROCESSING POLYNUCLEOTIDES

CROSS-REFERENCE TO RELATED APPLICATIONS

This application is a continuation of U.S. application Ser. No. 16/212,441, filed Dec. 6, 2018, which is a continuation of U.S. application Ser. No. 16/052,431, filed Aug. 1, 2018, which is a continuation-in-part of U.S. application Ser. No. 16/000,803, filed Jun. 5, 2018, which is a continuation of U.S. application Ser. No. 15/850,241, filed Dec. 21, 2017, which is a continuation of U.S. patent application Ser. No. 15/588,519, filed May 5, 2017, now U.S. Pat. No. 9,856,530, which is a continuation of U.S. patent application Ser. No. 15/376,582, filed Dec. 12, 2016, now U.S. Pat. No. 9,701,998, which is a continuation-in-part of U.S. patent application Ser. No. 14/104,650, filed on Dec. 12, 2013, now U.S. Pat. No. 9,567,631, which claims priority to U.S. Provisional Application No. 61/737,374, filed on Dec. 14, 2012; U.S. patent application Ser. No. 15/376,582 is also a continuation-in-part of U.S. patent application Ser. No. 14/250,701, filed on Apr. 11, 2014, which is a continuation of U.S. patent application Ser. No. 14/175,973, filed on Feb. 7, 2014, now U.S. Pat. No. 9,388,465, which claims priority to U.S. Provisional Application No. 61/844,804, filed on Jul. 10, 2013, U.S. Provisional Application No. 61/840,403, filed on Jun. 27, 2013, U.S. Provisional Application No. 61/800,223, filed on Mar. 15, 2013, and U.S. Provisional Application No. 61/762,435, filed on Feb. 8, 2013; U.S. application Ser. No. 16/052,431 is also a continuation-in-part of U.S. application Ser. No. 15/598,898, filed May 18, 2017, which is a continuation of U.S. application Ser. No. 14/624,468, filed Feb. 17, 2015, now U.S. Pat. No. 9,689,024, which is a division of U.S. patent application Ser. No. 13/966,150, filed Aug. 13, 2013, which claims priority to U.S. Provisional Application No. 61/683,192, filed Aug. 14, 2012, U.S. Provisional Application No. 61/737,374, filed Dec. 14, 2012, U.S. Provisional Application No. 61/762,435, filed Feb. 8, 2013, U.S. Provisional Application No. 61/800,223, filed Mar. 15, 2013, U.S. Provisional Application No. 61/840,403, filed Jun. 27, 2013, and U.S. Provisional Application No. 61/844,804, filed Jul. 10, 2013; this application is also a continuation-in-part of U.S. application Ser. No. 15/847,752, filed Dec. 19, 2017, which is a continuation of U.S. application Ser. No. 15/717,871, filed Sep. 27, 2017, now U.S. Pat. No. 9,951,386, which is a continuation-in-part of U.S. application Ser. No. 14/752,641, filed Jun. 26, 2015, which claims the benefit of U.S. Provisional Application No. 62/061,567, filed Oct. 8, 2014, and U.S. Provisional Application No. 62/017,558, filed Jun. 26, 2014; this application is also a continuation-in-part of U.S. application Ser. No. 16/052,486, which is a continuation-in-part of each of U.S. application Ser. No. 16/000,803 (which priority is recited above) and U.S. application Ser. No. 14/316,447, filed Jun. 26, 2014, which is a continuation-in-part of U.S. patent application Ser. No. 13/966,150 (which priority is recited above), filed Aug. 13, 2013, and a continuation-in-part of PCT/US13/54797, filed Aug. 13, 2013, which application claims the benefit of U.S. Provisional Application No. 61/683,192, filed on Aug. 14, 2012, U.S. Provisional Application No. 61/737,374, filed Dec. 14, 2012, U.S. Provisional Application No. 61/762,435, filed Feb. 8, 2013, U.S. Provisional Application No. 61/800,223, filed Mar. 15, 2013, U.S. Provisional Application No. 61/840,403 filed Jun. 27, 2013, and U.S. Provisional Application No. 61/844,804, filed Jul. 10, 2013; U.S. application Ser. No. 14/316,447 also claims

the benefit of U.S. Provisional Application No. 61/896,060, filed Oct. 26, 2013, U.S. Provisional Application No. 61/909,974, filed Nov. 27, 2013, U.S. Provisional Application No. 61/991,018, filed May 9, 2014, U.S. Provisional Application No. 61/937,344, filed Feb. 7, 2014, and U.S. Provisional Application No. 61/940,318, filed Feb. 14, 2014; each of which applications is entirely incorporated herein by reference for all purposes.

BACKGROUND

Significant advances in analyzing and characterizing biological and biochemical materials and systems have led to unprecedented advances in understanding the mechanisms of life, health, disease and treatment. Among these advances, technologies that target and characterize the genomic make up of biological systems have yielded some of the most groundbreaking results, including advances in the use and exploitation of genetic amplification technologies, and nucleic acid sequencing technologies.

Nucleic acid sequencing can be used to obtain information in a wide variety of biomedical contexts, including diagnostics, prognostics, biotechnology, and forensic biology. Sequencing may involve basic methods including Maxam-Gilbert sequencing and chain-termination methods, or de novo sequencing methods including shotgun sequencing and bridge PCR, or next-generation methods including polony sequencing, 454 pyrosequencing, Illumina sequencing, SOLiD sequencing, Ion Torrent semiconductor sequencing, Heli Scope single molecule sequencing, SMRT® sequencing, and others.

Despite these advances in biological characterization, many challenges still remain unaddressed, or relatively poorly addressed by the solutions currently being offered. The present disclosure provides novel solutions and approaches to addressing many of the shortcomings of existing technologies.

BRIEF SUMMARY

Provided herein are methods, compositions and systems for analyzing individual cells or small populations of cells, including the analysis and attribution of nucleic acids from and to these individual cells or cell populations.

An aspect of the disclosure provides a method of analyzing nucleic acids from cells that includes providing nucleic acids derived from an individual cell into a discrete partition; generating one or more first nucleic acid sequences derived from the nucleic acids within the discrete partition, which one or more first nucleic acid sequences have attached thereto oligonucleotides that comprise a common nucleic acid barcode sequence; generating a characterization of the one or more first nucleic acid sequences or one or more second nucleic acid sequences derived from the one or more first nucleic acid sequences, which one or more second nucleic acid sequences comprise the common barcode sequence; and identifying the one or more first nucleic acid sequences or one or more second nucleic acid sequences as being derived from the individual cell based, at least in part, upon a presence of the common nucleic acid barcode sequence in the generated characterization.

In some embodiments, the discrete partition is a discrete droplet. In some embodiments, the oligonucleotides are co-partitioned with the nucleic acids derived from the individual cell into the discrete partition. In some embodiments, at least 10,000, at least 100,000 or at least 500,000 of the

oligonucleotides are co-partitioned with the nucleic acids derived from the individual cell into the discrete partition.

In some embodiments, the oligonucleotides are provided attached to a bead, where each oligonucleotide on a bead comprises the same barcode sequence, and the bead is co-partitioned with the individual cell into the discrete partition. In some embodiments, the oligonucleotides are releasably attached to the bead. In some embodiments, the bead comprises a degradable bead. In some embodiments, prior to or during generating the one or more first nucleic acid sequences the method includes releasing the oligonucleotides from the bead via degradation of the bead. In some embodiments, prior to generating the characterization, the method includes releasing the one or more first nucleic acid sequences from the discrete partition.

In some embodiments, generating the characterization comprises sequencing the one or more first nucleic acid sequences or the one or more second nucleic acid sequences. The method may also include assembling a contiguous nucleic acid sequence for at least a portion of a genome of the individual cell from sequences of the one or more first nucleic acid sequences or the one or more second nucleic acid sequences. Moreover, the method may also include characterizing the individual cell based upon the nucleic acid sequence for at least a portion of the genome of the individual cell.

In some embodiments, the nucleic acids are released from the individual cell in the discrete partition. In some embodiments, the nucleic acids comprise ribonucleic acid (RNA), such as, for example, messenger RNA (mRNA). In some embodiments, generating one or more first nucleic acid sequences includes subjecting the nucleic acids to reverse transcription under conditions that yield the one or more first nucleic acid sequences. In some embodiments, the reverse transcription occurs in the discrete partition. In some embodiments, the oligonucleotides are provided in the discrete partition and include a poly-T sequence. In some embodiments, the reverse transcription comprises hybridizing the poly-T sequence to at least a portion of each of the nucleic acids and extending the poly-T sequence in template directed fashion. In some embodiments, the oligonucleotides include an anchoring sequence that facilitates hybridization of the poly-T sequence. In some embodiments, the oligonucleotides include a random priming sequence that can be, for example, a random hexamer. In some embodiments, the reverse transcription comprises hybridizing the random priming sequence to at least a portion of each of the nucleic acids and extending the random priming sequence in template directed fashion.

In some embodiments, a given one of the one or more first nucleic acid sequences has sequence complementarity to at least a portion of a given one of the nucleic acids. In some embodiments, the discrete partition at most includes the individual cell among a plurality of cells. In some embodiments, the oligonucleotides include a unique molecular sequence segment. In some embodiments, the method can include identifying an individual nucleic acid sequence of the one or more first nucleic acid sequences or of the one or more second nucleic acid sequences as derived from a given nucleic acid of the nucleic acids based, at least in part, upon a presence of the unique molecular sequence segment. In some embodiments, the method includes determining an amount of the given nucleic acid based upon a presence of the unique molecular sequence segment.

In some embodiments, the method includes, prior to generating the characterization, adding one or more additional sequences to the one or more first nucleic acid sequences to generate the one or more second nucleic acid sequences. In some embodiments, the method includes adding a first additional nucleic acid sequence to the one or more first nucleic acid sequences with the aid of a switch oligonucleotide. In some embodiments, the switch oligonucleotide hybridizes to at least a portion of the one or more first nucleic acid sequences and is extended in a template directed fashion to couple the first additional nucleic acid sequence to the one or more first nucleic acid sequences. In some embodiments, the method includes amplifying the one of more first nucleic acid sequences coupled to the first additional nucleic acid sequence. In some embodiments, the amplifying occurs in the discrete partition. In some embodiments, the amplifying occurs after releasing the one or more first nucleic acid sequences coupled to the first additional nucleic acid sequence from the discrete partition.

In some embodiments, after the amplifying, the method includes adding one or more second additional nucleic acid sequences to the one or more first nucleic acid sequences coupled to the first additional sequence to generate the one or more second nucleic acid sequences. In some embodiments, the adding the one or more second additional sequences includes removing a portion of each of the one or more first nucleic acid sequences coupled to the first additional nucleic acid sequence and coupling thereto the one or more second additional nucleic acid sequences. In some embodiments, the removing is completed via shearing of the one or more first nucleic acid sequences coupled (e.g., ligated) to the first additional nucleic acid sequence.

In some embodiments, prior to generating the characterization, the method includes subjecting the one or more first nucleic acid sequences to transcription to generate one or more RNA fragments. In some embodiments, the transcription occurs after releasing the one or more first nucleic acid sequences from the discrete partition. In some embodiments, the oligonucleotides include a T7 promoter sequence. In some embodiments, prior to generating the characterization, the method includes removing a portion of each of the one or more RNA sequences and coupling an additional sequence to the one or more RNA sequences. In some embodiments, prior to generating the characterization, the method includes subjecting the one or more RNA sequences coupled to the additional sequence to reverse transcription to generate the one or more second nucleic acid sequences. In some embodiments, prior to generating the characterization, the method includes amplifying the one or more second nucleic acid sequences. In some embodiments, prior to generating the characterization, the method includes subjecting the one or more RNA sequences to reverse transcription to generate one or more DNA sequences. In some embodiments, prior to generating the characterization, the method includes removing a portion of each of the one or more DNA sequences and coupling one or more additional sequences to the one or more DNA sequences to generate the one or more second nucleic acid sequences. In some embodiments, prior to generating the characterization, the method includes amplifying the one or more second nucleic acid sequences.

In some embodiments, the nucleic acids include complementary (cDNA) generated from reverse transcription of RNA from the individual cell. In some embodiments, the oligonucleotides include a priming sequence and are provided in the discrete partition. In some embodiments, the priming sequence includes a random N-mer. In some embodiments, generating the one or more first nucleic acid

sequences includes hybridizing the priming sequence to the cDNA and extending the priming sequence in template directed fashion.

In some embodiments, the discrete partition includes switch oligonucleotides comprising a complement sequence of the oligonucleotides. In some embodiments, generating the one or more first nucleic acid sequences includes hybridizing the switch oligonucleotides to at least a portion of nucleic acid fragments derived from the nucleic acids and extending the switch oligonucleotides in template directed fashion. In some embodiments, generating the one or more first nucleic acid sequences includes attaching the oligonucleotides to the one or more first nucleic acid sequences. In some embodiments, the one or more first nucleic acid sequences are nucleic acid fragments derived from the nucleic acids. In some embodiments, generating the one or more first nucleic acid sequences includes coupling (e.g., ligating) the oligonucleotides to the nucleic acids.

In some embodiments, a plurality of partitions comprises the discrete partition. In some embodiments, the plurality of partitions, on average, comprises less than one cell per partition. In some embodiments, less than 25% of partitions of the plurality of partitions do not comprise a cell. In some embodiments, the plurality of partitions comprises discrete partitions each having at least one partitioned cell. In some embodiments, fewer than 25%, fewer than 20%, fewer than 15%, fewer than 10%, fewer than 5% or fewer than 1% of the discrete partitions comprise more than one cell. In some embodiments, at least a subset of the discrete partitions comprises a bead. In some embodiments, at least 75%, at least 80%, at least 85%, at least 90%, at least 95% or at least 99% of the discrete partitions comprise at least one cell and at least one bead. In some embodiments, the discrete partitions include partitioned nucleic acid barcode sequences. In some embodiments, the discrete partitions include at least 1,000, at least 10,000, or at least 100,000 different partitioned nucleic acid barcode sequences. In some embodiments, the plurality of partitions comprises at least 1,000, at least 10,000 or at least 100,000 partitions.

In another aspect, the disclosure provides a method of characterizing cells in a population of a plurality of different cell types that includes providing nucleic acids from individual cells in the population into discrete partitions; attaching oligonucleotides that comprise a common nucleic acid barcode sequence to one or more fragments of the nucleic acids from the individual cells within the discrete partitions, where a plurality of different partitions comprise different common nucleic acid barcode sequences; and characterizing the one or more fragments of the nucleic acids from the plurality of discrete partitions, and attributing the one or more fragments to individual cells based, at least in part, upon the presence of a common barcode sequence; and characterizing a plurality of individual cells in the population based upon the characterization of the one or more fragments in the plurality of discrete partitions.

In some embodiments, the method includes fragmenting the nucleic acids. In some embodiments, the discrete partitions are droplets. In some embodiments, the characterizing the one or more fragments of the nucleic acids includes sequencing ribosomal deoxyribonucleic acid from the individual cells, and the characterizing the cells comprises identifying a cell genus, species, strain or variant. In some embodiments, the individual cells are derived from a microbiome sample. In some embodiments, the individual cells are derived from a human tissue sample. In some embodiments, the individual cells are derived from circulating cells in a mammal. In some embodiments, the individual cells are derived from a forensic sample. In some embodiments, the nucleic acids are released from the individual cells in the discrete partitions.

An additional aspect of the disclosure provides a method of characterizing an individual cell or population of cells that includes incubating a cell with a plurality of different cell surface feature binding group types, where each different cell surface binding group type is capable of binding to a different cell surface feature, and where each different cell surface binding group type comprises a reporter oligonucleotide associated therewith, under conditions that allow binding between one or more cell surface feature binding groups and its respective cell surface feature, if present; partitioning the cell into a partition that comprises a plurality of oligonucleotides comprising a barcode sequence; attaching the barcode sequence to oligonucleotide reporter groups present in the partition; sequencing the oligonucleotide reporter groups and attached barcodes; and characterizing cell surface features present on the cell based upon reporter oligonucleotides that are sequenced.

An additional aspect of the disclosure provides a composition comprising a plurality of partitions, each of the plurality of partitions comprising an individual cell and a population of oligonucleotides that comprise a common nucleic acid barcode sequence. In some embodiments, the plurality of partitions comprises droplets in an emulsion. In some embodiments, the population of oligonucleotides within each of the plurality of partitions is coupled to a bead disposed within each of the plurality of partitions. In some embodiments, the individual cell has associated therewith a plurality of different cell surface feature binding groups associated with their respective cell surface features and each different type of cell surface feature binding group includes an oligonucleotide reporter group comprising a different nucleotide sequence. In some embodiments, the plurality of different cell surface feature binding groups includes a plurality of different antibodies or antibody fragments having a binding affinity for a plurality of different cell surface features.

Additional aspects and advantages of the present disclosure will become readily apparent to those skilled in the art from the following detailed description, wherein only illustrative embodiments of the present disclosure are shown and described. As will be realized, the present disclosure is capable of other and different embodiments, and its several details are capable of modifications in various obvious respects, all without departing from the disclosure. Accordingly, the drawings and description are to be regarded as illustrative in nature, and not as restrictive.

INCORPORATION BY REFERENCE

All publications, patents, and patent applications mentioned in this specification are herein incorporated by reference to the same extent as if each individual publication, patent, or patent application was specifically and individually indicated to be incorporated by reference. To the extent publications and patents or patent applications incorporated by reference contradict the disclosure contained in the specification, the specification is intended to supersede and/or take precedence over any such contradictory material.

BRIEF DESCRIPTION OF THE DRAWINGS

The novel features of the invention are set forth with particularity in the appended claims. A better understanding

of the features and advantages of the present invention will be obtained by reference to the following detailed description that sets forth illustrative embodiments, in which the principles of the invention are utilized, and the accompanying drawings (also "Figure" and "FIG." herein), of which:

FIG. **1** schematically illustrates a microfluidic channel structure for partitioning individual or small groups of cells.

FIG. **2** schematically illustrates a microfluidic channel structure for co-partitioning cells and beads or microcapsules comprising additional reagents.

FIG. **3** schematically illustrates an example process for amplification and barcoding of cell's nucleic acids.

FIG. **4** provides a schematic illustration of use of barcoding of cell's nucleic acids in attributing sequence data to individual cells or groups of cells for use in their characterization.

FIG. **5** provides a schematic illustrating cells associated with labeled cell-binding ligands.

FIG. **6** provides a schematic illustration of an example workflow for performing RNA analysis using the methods described herein.

FIG. **7** provides a schematic illustration of an example barcoded oligonucleotide structure for use in analysis of ribonucleic (RNA) using the methods described herein.

FIG. **8** provides an image of individual cells co-partitioned along with individual barcode bearing beads

FIG. **9**A-E provides schematic illustration of example barcoded oligonucleotide structures for use in analysis of RNA and example operations for performing RNA analysis.

FIG. **10** provides schematic illustration of example barcoded oligonucleotide structure for use in example analysis of RNA and use of a sequence for in vitro transcription.

FIG. **11** provides schematic illustration of an example barcoded oligonucleotide structure for use in analysis of RNA and example operations for performing RNA analysis.

FIG. **12**A-B provides schematic illustration of example barcoded oligonucleotide structure for use in analysis of RNA.

FIG. **13**A-C provides illustrations of example yields from template switch reverse transcription and PCR in partitions.

FIG. **14**A-B provides illustrations of example yields from reverse transcription and cDNA amplification in partitions with various cell numbers.

FIG. **15** provides an illustration of example yields from cDNA synthesis and real-time quantitative PCR at various input cell concentrations and also the effect of varying primer concentration on yield at a fixed cell input concentration.

FIG. **16** provides an illustration of example yields from in vitro transcription.

FIG. **17** shows an example computer control system that is programmed or otherwise configured to implement methods provided herein.

DETAILED DESCRIPTION

While various embodiments of the invention have been shown and described herein, it will be obvious to those skilled in the art that such embodiments are provided by way of example only. Numerous variations, changes, and substitutions may occur to those skilled in the art without departing from the invention. It should be understood that various alternatives to the embodiments of the invention described herein may be employed.

Where values are described as ranges, it will be understood that such disclosure includes the disclosure of all possible sub-ranges within such ranges, as well as specific numerical values that fall within such ranges irrespective of whether a specific numerical value or specific sub-range is expressly stated.

I. SINGLE CELL ANALYSIS

Advanced nucleic acid sequencing technologies have yielded monumental results in sequencing biological materials, including providing substantial sequence information on individual organisms, and relatively pure biological samples. However, these systems have not proven effective at being able to identify and characterize sub-populations of cells in biological samples that may represent a smaller minority of the overall make up of the sample, but for which individualized sequence information could prove even more valuable.

Most nucleic acid sequencing technologies derive the nucleic acids that they sequence from collections of cells derived from tissue or other samples. The cells can be processed, en masse, to extract the genetic material that represents an average of the population of cells, which can then be processed into sequencing ready DNA libraries that are configured for a given sequencing technology. As will be appreciated, although often discussed in terms of DNA or nucleic acids, the nucleic acids derived from the cells may include DNA, or RNA, including, e.g., mRNA, total RNA, or the like, that may be processed to produce cDNA for sequencing, e.g., using any of a variety of RNA-seq methods. Following from this processing, absent a cell specific marker, attribution of genetic material as being contributed by a subset of cells or all cells in a sample is virtually impossible in such an ensemble approach.

In addition to the inability to attribute characteristics to particular subsets of populations of cells, such ensemble sample preparation methods also are, from the outset, predisposed to primarily identifying and characterizing the majority constituents in the sample of cells, and are not designed to be able to pick out the minority constituents, e.g., genetic material contributed by one cell, a few cells, or a small percentage of total cells in the sample. Likewise, where analyzing expression levels, e.g., of mRNA, an ensemble approach would be predisposed to presenting potentially grossly inaccurate data from cell populations that are non-homogeneous in terms of expression levels. In some cases, where expression is high in a small minority of the cells in an analyzed population, and absent in the majority of the cells of the population, an ensemble method would indicate low level expression for the entire population.

This original majority bias is further magnified, and even overwhelming, through processing operations used in building up the sequencing libraries from these samples. In particular, most next generation sequencing technologies rely upon the geometric amplification of nucleic acid fragments, such as the polymerase chain reaction, in order to produce sufficient DNA for the sequencing library. However, such geometric amplification is biased toward amplification of majority constituents in a sample, and may not preserve the starting ratios of such minority and majority components. By way of example, if a sample includes 95% DNA from a particular cell type in a sample, e.g., host tissue cells, and 5% DNA from another cell type, e.g., cancer cells, PCR based amplification can preferentially amplify the majority DNA in place of the minority DNA, both as a function of comparative exponential amplification (the repeated doubling of the higher concentration quickly outpaces that of the smaller fraction) and as a function of sequestration of

amplification reagents and resources (as the larger fraction is amplified, it preferentially utilizes primers and other amplification reagents).

While some of these difficulties may be addressed by utilizing different sequencing systems, such as single molecule systems that don't require amplification, the single molecule systems, as well as the ensemble sequencing methods of other next generation sequencing systems, can also have requirements for sufficiently large input DNA requirements. In particular, single molecule sequencing systems like the Pacific Biosciences SMRT Sequencing system can have sample input DNA requirements of from 500 nanograms (ng) to upwards of 10 micrograms (m), which is far larger than what can be derived from individual cells or even small subpopulations of cells. Likewise, other NGS systems can be optimized for starting amounts of sample DNA in the sample of from approximately 50 ng to about 1 μg.

II. COMPARTMENTALIZATION AND CHARACTERIZATION OF CELLS

Disclosed herein, however, are methods and systems for characterizing nucleic acids from small populations of cells, and in some cases, for characterizing nucleic acids from individual cells, especially in the context of larger populations of cells. The methods and systems provide advantages of being able to provide the attribution advantages of the non-amplified single molecule methods with the high throughput of the other next generation systems, with the additional advantages of being able to process and sequence extremely low amounts of input nucleic acids derivable from individual cells or small collections of cells.

In particular, the methods described herein compartmentalize the analysis of individual cells or small populations of cells, including e.g., nucleic acids from individual cells or small groups of cells, and then allow that analysis to be attributed back to the individual cell or small group of cells from which the nucleic acids were derived. This can be accomplished regardless of whether the cell population represents a 50/50 mix of cell types, a 90/10 mix of cell types, or virtually any ratio of cell types, as well as a complete heterogeneous mix of different cell types, or any mixture between these. Differing cell types may include cells or biologic organisms from different tissue types of an individual, from different individuals, from differing genera, species, strains, variants, or any combination of any or all of the foregoing. For example, differing cell types may include normal and tumor tissue from an individual, multiple different bacterial species, strains and/or variants from environmental, forensic, microbiome or other samples, or any of a variety of other mixtures of cell types.

In one aspect, the methods and systems described herein, provide for the compartmentalization, depositing or partitioning of the nucleic acid contents of individual cells from a sample material containing cells, into discrete compartments or partitions (referred to interchangeably herein as partitions), where each partition maintains separation of its own contents from the contents of other partitions. Unique identifiers, e.g., barcodes, may be previously, subsequently or concurrently delivered to the partitions that hold the compartmentalized or partitioned cells, in order to allow for the later attribution of the characteristics of the individual cells to the particular compartment.

As used herein, in some aspects, the partitions refer to containers or vessels (such as wells, microwells, tubes, through ports in nanoarray substrates, e.g., BioTrove nanoarrays, or other containers). In many some aspects, however, the compartments or partitions comprise partitions that are flowable within fluid streams. These partitions may be comprised of, e.g., microcapsules or micro-vesicles that have an outer barrier surrounding an inner fluid center or core, or they may be a porous matrix that is capable of entraining and/or retaining materials within its matrix. In some aspects, however, these partitions comprise droplets of aqueous fluid within a non-aqueous continuous phase, e.g., an oil phase. A variety of different vessels are described in, for example, U.S. patent application Ser. No. 13/966,150, filed Aug. 13, 2013, the full disclosure of which is incorporated herein by reference in its entirety for all purposes. Likewise, emulsion systems for creating stable droplets in non-aqueous or oil continuous phases are described in detail in, e.g., U.S. Patent Publication No. 2010/0105112, the full disclosure of which is incorporated herein by reference in its entirety for all purposes.

In the case of droplets in an emulsion, allocating individual cells to discrete partitions may generally be accomplished by introducing a flowing stream of cells in an aqueous fluid into a flowing stream of a non-aqueous fluid, such that droplets are generated at the junction of the two streams. By providing the aqueous cell-containing stream at a certain concentration level of cells, one can control the level of occupancy of the resulting partitions in terms of numbers of cells. In some cases, where single cell partitions are desired, it may be desirable to control the relative flow rates of the fluids such that, on average, the partitions contain less than one cell per partition, in order to ensure that those partitions that are occupied, are primarily singly occupied. Likewise, one may wish to control the flow rate to provide that a higher percentage of partitions are occupied, e.g., allowing for only a small percentage of unoccupied partitions. In some aspects, the flows and channel architectures are controlled as to ensure a desired number of singly occupied partitions, less than a certain level of unoccupied partitions and less than a certain level of multiply occupied partitions.

In many cases, the systems and methods are used to ensure that the substantial majority of occupied partitions (partitions containing one or more microcapsules) include no more than 1 cell per occupied partition. In some cases, the partitioning process is controlled such that fewer than 25% of the occupied partitions contain more than one cell, and in many cases, fewer than 20% of the occupied partitions have more than one cell, while in some cases, fewer than 10% or even fewer than 5% of the occupied partitions include more than one cell per partition.

Additionally or alternatively, in many cases, it is desirable to avoid the creation of excessive numbers of empty partitions. While this may be accomplished by providing sufficient numbers of cells into the partitioning zone, the poissonian distribution would expectedly increase the number of partitions that would include multiple cells. As such, in accordance with aspects described herein, the flow of one or more of the cells, or other fluids directed into the partitioning zone are controlled such that, in many cases, no more than 50% of the generated partitions are unoccupied, i.e., including less than 1 cell, no more than 25% of the generated partitions, no more than 10% of the generated partitions, may be unoccupied. Further, in some aspects, these flows are controlled so as to present non-poissonian distribution of single occupied partitions while providing lower levels of unoccupied partitions. Restated, in some aspects, the above noted ranges of unoccupied partitions can be achieved while still providing any of the single occupancy rates described

above. For example, in many cases, the use of the systems and methods described herein creates resulting partitions that have multiple occupancy rates of from less than 25%, less than 20%, less than 15%, less than 10%, and in many cases, less than 5%, while having unoccupied partitions of from less than 50%, less than 40%, less than 30%, less than 20%, less than 10%, and in some cases, less than 5%.

As will be appreciated, the above-described occupancy rates are also applicable to partitions that include both cells and beads carrying the barcode oligonucleotides. In particular, in some aspects, a substantial percentage of the overall occupied partitions will include both a bead and a cell. In particular, it may be desirable to provide that at least 50% of the partitions are occupied by at least one cell and at least one bead, or at least 75% of the partitions may be so occupied, or even at least 80% or at least 90% of the partitions may be so occupied. Further, in those cases where it is desired to provide a single cell and a single bead within a partition, at least 50% of the partitions can be so occupied, at least 60%, at least 70%, at least 80% or even at least 90% of the partitions can be so occupied.

Although described in terms of providing substantially singly occupied partitions, above, in certain cases, it is desirable to provide multiply occupied partitions, e.g., containing two, three, four or more cells and/or beads within a single partition. Accordingly, as noted above, the flow characteristics of the cell and/or bead containing fluids and partitioning fluids may be controlled to provide for such multiply occupied partitions. In particular, the flow parameters may be controlled to provide a desired occupancy rate at greater than 50% of the partitions, greater than 75%, and in some cases greater than 80%, 90%, 95%, or higher.

Additionally, in many cases, the multiple beads within a single partition may comprise different reagents associated therewith. In such cases, it may be advantageous to introduce different beads into a common channel or droplet generation junction, from different bead sources, i.e., containing different associated reagents, through different channel inlets into such common channel or droplet generation junction. In such cases, the flow and frequency of the different beads into the channel or junction may be controlled to provide for the desired ratio of microcapsules from each source, while ensuring the desired pairing or combination of such beads into a partition with the desired number of cells.

The partitions described herein are often characterized by having extremely small volumes, e.g., less than 10 μL, less than 5 μL, less than 1 μL, less than 900 picoliters (pL), less than 800 pL, less than 700 pL, less than 600 pL, less than 500 pL, less than 400 pL, less than 300 pL, less than 200 pL, less than 100 pL, less than 50 pL, less than 20 pL, less than 10 pL, less than 1 pL, less than 500 nanoliters (nL), or even less than 100 nL, 50 nL, or even less.

For example, in the case of droplet based partitions, the droplets may have overall volumes that are less than 1000 pL, less than 900 pL, less than 800 pL, less than 700 pL, less than 600 pL, less than 500 pL, less than 400 pL, less than 300 pL, less than 200 pL, less than 100 pL, less than 50 pL, less than 20 pL, less than 10 pL, or even less than 1 pL. Where co-partitioned with beads, it will be appreciated that the sample fluid volume, e.g., including co-partitioned cells, within the partitions may be less than 90% of the above described volumes, less than 80%, less than 70%, less than 60%, less than 50%, less than 40%, less than 30%, less than 20%, or even less than 10% the above described volumes.

As is described elsewhere herein, partitioning species may generate a population of partitions. In such cases, any suitable number of partitions can be generated to generate the population of partitions. For example, in a method described herein, a population of partitions may be generated that comprises at least about 1,000 partitions, at least about 5,000 partitions, at least about 10,000 partitions, at least about 50,000 partitions, at least about 100,000 partitions, at least about 500,000 partitions, at least about 1,000,000 partitions, at least about 5,000,000 partitions at least about 10,000,000 partitions, at least about 50,000,000 partitions, at least about 100,000,000 partitions, at least about 500,000,000 partitions or at least about 1,000,000,000 partitions. Moreover, the population of partitions may comprise both unoccupied partitions (e.g., empty partitions) and occupied partitions

In certain cases, microfluidic channel networks are particularly suited for generating partitions as described herein. Examples of such microfluidic devices include those described in detail in Provisional U.S. Patent Application No. 61/977,804, filed Apr. 4, 2014, the full disclosure of which is incorporated herein by reference in its entirety for all purposes. Alternative mechanisms may also be employed in the partitioning of individual cells, including porous membranes through which aqueous mixtures of cells are extruded into non-aqueous fluids. Such systems are generally available from, e.g., Nanomi, Inc.

An example of a simplified microfluidic channel structure for partitioning individual cells is illustrated in FIG. **1**. As described elsewhere herein, in some cases, the majority of occupied partitions include no more than one cell per occupied partition and, in some cases, some of the generated partitions are unoccupied. In some cases, though, some of the occupied partitions may include more than one cell. In some cases, the partitioning process may be controlled such that fewer than 25% of the occupied partitions contain more than one cell, and in many cases, fewer than 20% of the occupied partitions have more than one cell, while in some cases, fewer than 10% or even fewer than 5% of the occupied partitions include more than one cell per partition. As shown, the channel structure can include channel segments **102**, **104**, **106** and **108** communicating at a channel junction **110**. In operation, a first aqueous fluid **112** that includes suspended cells **114**, may be transported along channel segment **102** into junction **110**, while a second fluid **116** that is immiscible with the aqueous fluid **112** is delivered to the junction **110** from channel segments **104** and **106** to create discrete droplets **118** of the aqueous fluid including individual cells **114**, flowing into channel segment **108**.

In some aspects, this second fluid **116** comprises an oil, such as a fluorinated oil, that includes a fluorosurfactant for stabilizing the resulting droplets, e.g., inhibiting subsequent coalescence of the resulting droplets. Examples of particularly useful partitioning fluids and fluorosurfactants are described for example, in U.S. Patent Publication No. 2010/0105112, the full disclosure of which is hereby incorporated herein by reference in its entirety for all purposes.

In other aspects, in addition to or as an alternative to droplet based partitioning, cells may be encapsulated within a microcapsule that comprises an outer shell or layer or porous matrix in which is entrained one or more individual cells or small groups of cells, and may include other reagents. Encapsulation of cells may be carried out by a variety of processes. In general, such processes combine an aqueous fluid containing the cells to be analyzed with a polymeric precursor material that may be capable of being formed into a gel or other solid or semi-solid matrix upon application of a particular stimulus to the polymer precursor. Such stimuli include, e.g., thermal stimuli (either heating or

cooling), photo-stimuli (e.g., through photo-curing), chemical stimuli (e.g., through crosslinking, polymerization initiation of the precursor (e.g., through added initiators), or the like.

Preparation of microcapsules comprising cells may be carried out by a variety of methods. For example, air knife droplet or aerosol generators may be used to dispense droplets of precursor fluids into gelling solutions in order to form microcapsules that include individual cells or small groups of cells. Likewise, membrane based encapsulation systems, such as those available from, e.g., Nanomi, Inc., may be used to generate microcapsules as described herein. In some aspects, microfluidic systems like that shown in FIG. **1** may be readily used in encapsulating cells as described herein. In particular, and with reference to FIG. **1**, the aqueous fluid comprising the cells and the polymer precursor material is flowed into channel junction **110**, where it is partitioned into droplets **118** comprising the individual cells **114**, through the flow of non-aqueous fluid **116**. In the case of encapsulation methods, non-aqueous fluid **116** may also include an initiator to cause polymerization and/or crosslinking of the polymer precursor to form the microcapsule that includes the entrained cells. Examples of particularly useful polymer precursor/initiator pairs include those described in, e.g., U.S. Patent Application Nos. 61/940,318, filed Feb. 7, 2014, 61/991,018, Filed May 9, 2014, and U.S. patent application Ser. No. 14/316,383, filed Jun. 26, 2014, the full disclosures of which are hereby incorporated herein by reference in their entireties for all purposes.

For example, in the case where the polymer precursor material comprises a linear polymer material, e.g., a linear polyacrylamide, PEG, or other linear polymeric material, the activation agent may comprise a cross-linking agent, or a chemical that activates a cross-linking agent within the formed droplets. Likewise, for polymer precursors that comprise polymerizable monomers, the activation agent may comprise a polymerization initiator. For example, in certain cases, where the polymer precursor comprises a mixture of acrylamide monomer with a N,N'-bis-(acryloyl) cystamine (BAC) comonomer, an agent such as tetraethylmethylenediamine (TEMED) may be provided within the second fluid streams in channel segments **104** and **106**, which initiates the copolymerization of the acrylamide and BAC into a cross-linked polymer network or, hydrogel.

Upon contact of the second fluid stream **116** with the first fluid stream **112** at junction **110** in the formation of droplets, the TEMED may diffuse from the second fluid **116** into the aqueous first fluid **112** comprising the linear polyacrylamide, which will activate the crosslinking of the polyacrylamide within the droplets, resulting in the formation of the gel, e.g., hydrogel, microcapsules **118**, as solid or semi-solid beads or particles entraining the cells **114**. Although described in terms of polyacrylamide encapsulation, other 'activatable' encapsulation compositions may also be employed in the context of the methods and compositions described herein. For example, formation of alginate droplets followed by exposure to divalent metal ions, e.g., Ca2+, can be used as an encapsulation process using the described processes. Likewise, agarose droplets may also be transformed into capsules through temperature based gelling, e.g., upon cooling, or the like. As will be appreciated, in some cases, encapsulated cells can be selectively releasable from the microcapsule, e.g., through passage of time, or upon application of a particular stimulus, that degrades the microcapsule sufficiently to allow the cell, or its contents to be released from the microcapsule, e.g., into an additional partition, such as a droplet. For example, in the case of the polyacrylamide polymer described above, degradation of the microcapsule may be accomplished through the introduction of an appropriate reducing agent, such as DTT or the like, to cleave disulfide bonds that cross link the polymer matrix (See, e.g., U.S. Provisional Patent Application Nos. 61/940,318, filed Feb. 7, 2014, 61/991,018, Filed May 9, 2014, and U.S. patent application Ser. No. 14/316,383, filed Jun. 26, 2014, the full disclosures of which are hereby incorporated herein by reference in their entirety for all purposes.

As will be appreciated, encapsulated cells or cell populations provide certain potential advantages of being storable, and more portable than droplet based partitioned cells. Furthermore, in some cases, it may be desirable to allow cells to be analyzed to incubate for a select period of time, in order to characterize changes in such cells over time, either in the presence or absence of different stimuli. In such cases, encapsulation of individual cells may allow for longer incubation than simple partitioning in emulsion droplets, although in some cases, droplet partitioned cells may also be incubated form different periods of time, e.g., at least 10 seconds, at least 30 seconds, at least 1 minute, at least 5 minutes, at least 10 minutes, at least 30 minutes, at least 1 hour, at least 2 hours, at least 5 hours, or at least 10 hours or more. As alluded to above, the encapsulation of cells may constitute the partitioning of the cells into which other reagents are co-partitioned. Alternatively, encapsulated cells may be readily deposited into other partitions, e.g., droplets, as described above.

In accordance with certain aspects, the cells may be partitioned along with lysis reagents in order to release the contents of the cells within the partition. In such cases, the lysis agents can be contacted with the cell suspension concurrently with, or immediately prior to the introduction of the cells into the partitioning junction/droplet generation zone, e.g., through an additional channel or channels upstream of channel junction **110**. Examples of lysis agents include bioactive reagents, such as lysis enzymes that are used for lysis of different cell types, e.g., gram positive or negative bacteria, plants, yeast, mammalian, etc., such as lysozymes, achromopeptidase, lysostaphin, labiase, kitalase, lyticase, and a variety of other lysis enzymes available from, e.g., Sigma-Aldrich, Inc. (St Louis, Mo.), as well as other commercially available lysis enzymes. Other lysis agents may additionally or alternatively be co-partitioned with the cells to cause the release of the cell's contents into the partitions. For example, in some cases, surfactant based lysis solutions may be used to lyse cells, although these may be less desirable for emulsion based systems where the surfactants can interfere with stable emulsions. In some cases, lysis solutions may include non-ionic surfactants such as, for example, TritonX-100 and Tween 20. In some cases, lysis solutions may include ionic surfactants such as, for example, sarcosyl and sodium dodecyl sulfate (SDS). Similarly, lysis methods that employ other methods may be used, such as electroporation, thermal, acoustic or mechanical cellular disruption may also be used in certain cases, e.g., non-emulsion based partitioning such as encapsulation of cells that may be in addition to or in place of droplet partitioning, where any pore size of the encapsulate is sufficiently small to retain nucleic acid fragments of a desired size, following cellular disruption.

In addition to the lysis agents co-partitioned with the cells described above, other reagents can also be co-partitioned with the cells, including, for example, DNase and RNase inactivating agents or inhibitors, such as proteinase K, chelating agents, such as EDTA, and other reagents

employed in removing or otherwise reducing negative activity or impact of different cell lysate components on subsequent processing of nucleic acids. In addition, in the case of encapsulated cells, the cells may be exposed to an appropriate stimulus to release the cells or their contents from a co-partitioned microcapsule. For example, in some cases, a chemical stimulus may be co-partitioned along with an encapsulated cell to allow for the degradation of the microcapsule and release of the cell or its contents into the larger partition. In some cases, this stimulus may be the same as the stimulus described elsewhere herein for release of oligonucleotides from their respective bead or partition. In alternative aspects, this may be a different and non-overlapping stimulus, in order to allow an encapsulated cell to be released into a partition at a different time from the release of oligonucleotides into the same partition.

Additional reagents may also be co-partitioned with the cells, such as endonucleases to fragment the cell's DNA, DNA polymerase enzymes and dNTPs used to amplify the cell's nucleic acid fragments and to attach the barcode oligonucleotides to the amplified fragments. Additional reagents may also include reverse transcriptase enzymes, including enzymes with terminal transferase activity, primers and oligonucleotides, and switch oligonucleotides (also referred to herein as "switch oligos") which can be used for template switching. In some cases, template switching can be used to increase the length of a cDNA. In one example of template switching, cDNA can be generated from reverse transcription of a template, e.g., cellular mRNA, where a reverse transcriptase with terminal transferase activity can add additional nucleotides, e.g., polyC, to the cDNA that are not encoded by the template, such, as at an end of the cDNA. Switch oligos can include sequences complementary to the additional nucleotides, e.g. polyG. The additional nucleotides (e.g., polyC) on the cDNA can hybridize to the sequences complementary to the additional nucleotides (e.g., polyG) on the switch oligo, whereby the switch oligo can be used by the reverse transcriptase as template to further extend the cDNA. Switch oligos may comprise deoxyribonucleic acids, ribonucleic acids, modified nucleic acids including locked nucleic acids (LNA), or any combination.

In some cases, the length of a switch oligo may be 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99, 100, 101, 102, 103, 104, 105, 106, 107, 108, 109, 110, 111, 112, 113, 114, 115, 116, 117, 118, 119, 120, 121, 122, 123, 124, 125, 126, 127, 128, 129, 130, 131, 132, 133, 134, 135, 136, 137, 138, 139, 140, 141, 142, 143, 144, 145, 146, 147, 148, 149, 150, 151, 152, 153, 154, 155, 156, 157, 158, 159, 160, 161, 162, 163, 164, 165, 166, 167, 168, 169, 170, 171, 172, 173, 174, 175, 176, 177, 178, 179, 180, 181, 182, 183, 184, 185, 186, 187, 188, 189, 190, 191, 192, 193, 194, 195, 196, 197, 198, 199, 200, 201, 202, 203, 204, 205, 206, 207, 208, 209, 210, 211, 212, 213, 214, 215, 216, 217, 218, 219, 220, 221, 222, 223, 224, 225, 226, 227, 228, 229, 230, 231, 232, 233, 234, 235, 236, 237, 238, 239, 240, 241, 242, 243, 244, 245, 246, 247, 248, 249, 250 nucleotides or longer.

In some cases, the length of a switch oligo may be at least 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99, 100, 101, 102, 103, 104, 105, 106, 107, 108, 109, 110, 111, 112, 113, 114, 115, 116, 117, 118, 119, 120, 121, 122, 123, 124, 125, 126, 127, 128, 129, 130, 131, 132, 133, 134, 135, 136, 137, 138, 139, 140, 141, 142, 143, 144, 145, 146, 147, 148, 149, 150, 151, 152, 153, 154, 155, 156, 157, 158, 159, 160, 161, 162, 163, 164, 165, 166, 167, 168, 169, 170, 171, 172, 173, 174, 175, 176, 177, 178, 179, 180, 181, 182, 183, 184, 185, 186, 187, 188, 189, 190, 191, 192, 193, 194, 195, 196, 197, 198, 199, 200, 201, 202, 203, 204, 205, 206, 207, 208, 209, 210, 211, 212, 213, 214, 215, 216, 217, 218, 219, 220, 221, 222, 223, 224, 225, 226, 227, 228, 229, 230, 231, 232, 233, 234, 235, 236, 237, 238, 239, 240, 241, 242, 243, 244, 245, 246, 247, 248, 249 or 250 nucleotides or longer.

In some cases, the length of a switch oligo may be at most 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99, 100, 101, 102, 103, 104, 105, 106, 107, 108, 109, 110, 111, 112, 113, 114, 115, 116, 117, 118, 119, 120, 121, 122, 123, 124, 125, 126, 127, 128, 129, 130, 131, 132, 133, 134, 135, 136, 137, 138, 139, 140, 141, 142, 143, 144, 145, 146, 147, 148, 149, 150, 151, 152, 153, 154, 155, 156, 157, 158, 159, 160, 161, 162, 163, 164, 165, 166, 167, 168, 169, 170, 171, 172, 173, 174, 175, 176, 177, 178, 179, 180, 181, 182, 183, 184, 185, 186, 187, 188, 189, 190, 191, 192, 193, 194, 195, 196, 197, 198, 199, 200, 201, 202, 203, 204, 205, 206, 207, 208, 209, 210, 211, 212, 213, 214, 215, 216, 217, 218, 219, 220, 221, 222, 223, 224, 225, 226, 227, 228, 229, 230, 231, 232, 233, 234, 235, 236, 237, 238, 239, 240, 241, 242, 243, 244, 245, 246, 247, 248, 249 or 250 nucleotides.

Once the contents of the cells are released into their respective partitions, the nucleic acids contained therein may be further processed within the partitions. In accordance with the methods and systems described herein, the nucleic acid contents of individual cells are generally provided with unique identifiers such that, upon characterization of those nucleic acids they may be attributed as having been derived from the same cell or cells. The ability to attribute characteristics to individual cells or groups of cells is provided by the assignment of unique identifiers specifically to an individual cell or groups of cells, which is another advantageous aspect of the methods and systems described herein. In particular, unique identifiers, e.g., in the form of nucleic acid barcodes are assigned or associated with individual cells or populations of cells, in order to tag or label the cell's components (and as a result, its characteristics) with the unique identifiers. These unique identifiers are then used to attribute the cell's components and characteristics to an individual cell or group of cells. In some aspects, this is carried out by co-partitioning the individual cells or groups of cells with the unique identifiers. In some aspects, the unique identifiers are provided in the form of oligonucleotides that comprise nucleic acid barcode sequences that may be attached to or otherwise associated with the nucleic acid contents of individual cells, or to other components of the cells, and particularly to fragments of those nucleic acids. The oligonucleotides are partitioned such that as between oligonucleotides in a given partition, the nucleic acid barcode sequences contained therein are the same, but as between different partitions, the oligonucleotides can, and do have differing barcode sequences, or at least represent a large number of different barcode sequences across all of the

partitions in a given analysis. In some aspects, only one nucleic acid barcode sequence can be associated with a given partition, although in some cases, two or more different barcode sequences may be present.

The nucleic acid barcode sequences can include from 6 to about 20 or more nucleotides within the sequence of the oligonucleotides. In some cases, the length of a barcode sequence may be 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20 nucleotides or longer. In some cases, the length of a barcode sequence may be at least 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20 nucleotides or longer. In some cases, the length of a barcode sequence may be at most 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20 nucleotides or shorter. These nucleotides may be completely contiguous, i.e., in a single stretch of adjacent nucleotides, or they may be separated into two or more separate subsequences that are separated by 1 or more nucleotides. In some cases, separated barcode subsequences can be from about 4 to about 16 nucleotides in length. In some cases, the barcode subsequence may be 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16 nucleotides or longer. In some cases, the barcode subsequence may be at least 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16 nucleotides or longer. In some cases, the barcode subsequence may be at most 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16 nucleotides or shorter.

The co-partitioned oligonucleotides can also comprise other functional sequences useful in the processing of the nucleic acids from the co-partitioned cells. These sequences include, e.g., targeted or random/universal amplification primer sequences for amplifying the genomic DNA from the individual cells within the partitions while attaching the associated barcode sequences, sequencing primers or primer recognition sites, hybridization or probing sequences, e.g., for identification of presence of the sequences or for pulling down barcoded nucleic acids, or any of a number of other potential functional sequences. Again, co-partitioning of oligonucleotides and associated barcodes and other functional sequences, along with sample materials is described in, for example, U.S. Patent Application Nos. 61/940,318, filed Feb. 7, 2014, 61/991,018, filed May 9, 2014, and U.S. patent application Ser. No. 14/316,383, filed Jun. 26, 2014, as well as U.S. patent application Ser. No. 14/175,935, filed Feb. 7, 2014, the full disclosures of which are incorporated herein by reference in their entireties for all purposes. As will be appreciated other mechanisms of co-partitioning oligonucleotides may also be employed, including, e.g., coalescence of two or more droplets, where one droplet contains oligonucleotides, or microdispensing of oligonucleotides into partitions, e.g., droplets within microfluidic systems.

Briefly, in one example, beads, microparticles or microcapsules are provided that each include large numbers of the above described oligonucleotides releasably attached to the beads, where all of the oligonucleotides attached to a particular bead will include the same nucleic acid barcode sequence, but where a large number of diverse barcode sequences are represented across the population of beads used. In particularly useful examples, hydrogel beads, e.g., comprising polyacrylamide polymer matrices, are used as a solid support and delivery vehicle for the oligonucleotides into the partitions, as they are capable of carrying large numbers of oligonucleotide molecules, and may be configured to release those oligonucleotides upon exposure to a particular stimulus, as described elsewhere herein. In some cases, the population of beads will provide a diverse barcode sequence library that includes at least 1,000 different barcode sequences, at least 5,000 different barcode sequences, at least 10,000 different barcode sequences, at least at least 50,000 different barcode sequences, at least 100,000 different barcode sequences, at least 1,000,000 different barcode sequences, at least 5,000,000 different barcode sequences, or at least 10,000,000 different barcode sequences. Additionally, each bead can be provided with large numbers of oligonucleotide molecules attached. In particular, the number of molecules of oligonucleotides including the barcode sequence on an individual bead can be at least 1,000 oligonucleotide molecules, at least 5,000 oligonucleotide molecules, at least 10,000 oligonucleotide molecules, at least 50,000 oligonucleotide molecules, at least 100,000 oligonucleotide molecules, at least 500,000 oligonucleotides, at least 1,000,000 oligonucleotide molecules, at least 5,000,000 oligonucleotide molecules, at least 10,000,000 oligonucleotide molecules, at least 50,000,000 oligonucleotide molecules, at least 100,000,000 oligonucleotide molecules, and in some cases at least 1 billion oligonucleotide molecules.

Moreover, when the population of beads is partitioned, the resulting population of partitions can also include a diverse barcode library that includes at least 1,000 different barcode sequences, at least 5,000 different barcode sequences, at least 10,000 different barcode sequences, at least at least 50,000 different barcode sequences, at least 100,000 different barcode sequences, at least 1,000,000 different barcode sequences, at least 5,000,000 different barcode sequences, or at least 10,000,000 different barcode sequences. Additionally, each partition of the population can include at least 1,000 oligonucleotide molecules, at least 5,000 oligonucleotide molecules, at least 10,000 oligonucleotide molecules, at least 50,000 oligonucleotide molecules, at least 100,000 oligonucleotide molecules, at least 500,000 oligonucleotides, at least 1,000,000 oligonucleotide molecules, at least 5,000,000 oligonucleotide molecules, at least 10,000,000 oligonucleotide molecules, at least 50,000,000 oligonucleotide molecules, at least 100,000,000 oligonucleotide molecules, and in some cases at least 1 billion oligonucleotide molecules.

In some cases, it may be desirable to incorporate multiple different barcodes within a given partition, either attached to a single or multiple beads within the partition. For example, in some cases, a mixed, but known barcode sequences set may provide greater assurance of identification in the subsequent processing, e.g., by providing a stronger address or attribution of the barcodes to a given partition, as a duplicate or independent confirmation of the output from a given partition.

The oligonucleotides are releasable from the beads upon the application of a particular stimulus to the beads. In some cases, the stimulus may be a photo-stimulus, e.g., through cleavage of a photo-labile linkage that releases the oligonucleotides. In other cases, a thermal stimulus may be used, where elevation of the temperature of the beads environment will result in cleavage of a linkage or other release of the oligonucleotides form the beads. In still other cases, a chemical stimulus is used that cleaves a linkage of the oligonucleotides to the beads, or otherwise results in release of the oligonucleotides from the beads. Examples of this type of system are described in U.S. patent application Ser. No. 13/966,150, filed Aug. 13, 2013, as well as U.S. Provisional Patent Application Nos. 61/940,318, filed Feb. 7, 2014, 61/991,018, Filed May 9, 2014, and U.S. patent application Ser. No. 14/316,383, filed Jun. 26, 2014, the full disclosures of which are hereby incorporated herein by reference n their entireties for all purposes. In one case, such compositions include the polyacrylamide matrices described

above for encapsulation of cells, and may be degraded for release of the attached oligonucleotides through exposure to a reducing agent, such as DTT.

In accordance with the methods and systems described herein, the beads including the attached oligonucleotides are co-partitioned with the individual cells, such that a single bead and a single cell are contained within an individual partition. As noted above, while single cell/single bead occupancy is the most desired state, it will be appreciated that multiply occupied partitions (either in terms of cells, beads or both), or unoccupied partitions (either in terms of cells, beads or both) will often be present. An example of a microfluidic channel structure for co-partitioning cells and beads comprising barcode oligonucleotides is schematically illustrated in FIG. **2**. As described elsewhere herein, in some aspects, a substantial percentage of the overall occupied partitions will include both a bead and a cell and, in some cases, some of the partitions that are generated will be unoccupied. In some cases, some of the partitions may have beads and cells that are not partitioned 1:1. In some cases, it may be desirable to provide multiply occupied partitions, e.g., containing two, three, four or more cells and/or beads within a single partition. As shown, channel segments **202**, **204**, **206**, **208** and **210** are provided in fluid communication at channel junction **212**. An aqueous stream comprising the individual cells **214**, is flowed through channel segment **202** toward channel junction **212**. As described above, these cells may be suspended within an aqueous fluid, or may have been pre-encapsulated, prior to the partitioning process.

Concurrently, an aqueous stream comprising the barcode carrying beads **216**, is flowed through channel segment **204** toward channel junction **212**. A non-aqueous partitioning fluid **216** is introduced into channel junction **212** from each of side channels **206** and **208**, and the combined streams are flowed into outlet channel **210**. Within channel junction **212**, the two combined aqueous streams from channel segments **202** and **204** are combined, and partitioned into droplets **218**, that include co-partitioned cells **214** and beads **216**. As noted previously, by controlling the flow characteristics of each of the fluids combining at channel junction **212**, as well as controlling the geometry of the channel junction, one can optimize the combination and partitioning to achieve a desired occupancy level of beads, cells or both, within the partitions **218** that are generated.

In some cases, lysis agents, e.g., cell lysis enzymes, may be introduced into the partition with the bead stream, e.g., flowing through channel segment **204**, such that lysis of the cell only commences at or after the time of partitioning. Additional reagents may also be added to the partition in this configuration, such as endonucleases to fragment the cell's DNA, DNA polymerase enzyme and dNTPs used to amplify the cell's nucleic acid fragments and to attach the barcode oligonucleotides to the amplified fragments. As noted above, in many cases, a chemical stimulus, such as DTT, may be used to release the barcodes from their respective beads into the partition. In such cases, it may be particularly desirable to provide the chemical stimulus along with the cell-containing stream in channel segment **202**, such that release of the barcodes only occurs after the two streams have been combined, e.g., within the partitions **218**. Where the cells are encapsulated, however, introduction of a common chemical stimulus, e.g., that both releases the oligonucleotides form their beads, and releases cells from their microcapsules may generally be provided from a separate additional side channel (not shown) upstream of or connected to channel junction **212**.

As will be appreciated, a number of other reagents may be co-partitioned along with the cells, beads, lysis agents and chemical stimuli, including, for example, protective reagents, like proteinase K, chelators, nucleic acid extension, replication, transcription or amplification reagents such as polymerases, reverse transcriptases, transposases which can be used for transposon based methods (e.g., Nextera), nucleoside triphosphates or NTP analogues, primer sequences and additional cofactors such as divalent metal ions used in such reactions, ligation reaction reagents, such as ligase enzymes and ligation sequences, dyes, labels, or other tagging reagents.

The channel networks, e.g., as described herein, can be fluidly coupled to appropriate fluidic components. For example, the inlet channel segments, e.g., channel segments **202**, **204**, **206** and **208** are fluidly coupled to appropriate sources of the materials they are to deliver to channel junction **212**. For example, channel segment **202** will be fluidly coupled to a source of an aqueous suspension of cells **214** to be analyzed, while channel segment **204** would be fluidly coupled to a source of an aqueous suspension of beads **216**. Channel segments **206** and **208** would then be fluidly connected to one or more sources of the non-aqueous fluid. These sources may include any of a variety of different fluidic components, from simple reservoirs defined in or connected to a body structure of a microfluidic device, to fluid conduits that deliver fluids from off-device sources, manifolds, or the like. Likewise, the outlet channel segment **210** may be fluidly coupled to a receiving vessel or conduit for the partitioned cells. Again, this may be a reservoir defined in the body of a microfluidic device, or it may be a fluidic conduit for delivering the partitioned cells to a subsequent process operation, instrument or component.

FIG. **8** shows images of individual Jurkat cells co-partitioned along with barcode oligonucleotide containing beads in aqueous droplets in an aqueous in oil emulsion. As illustrated, individual cells may be readily co-partitioned with individual beads. As will be appreciated, optimization of individual cell loading may be carried out by a number of methods, including by providing dilutions of cell populations into the microfluidic system in order to achieve the desired cell loading per partition as described elsewhere herein.

In operation, once lysed, the nucleic acid contents of the individual cells are then available for further processing within the partitions, including, e.g., fragmentation, amplification and barcoding, as well as attachment of other functional sequences. As noted above, fragmentation may be accomplished through the co-partitioning of shearing enzymes, such as endonucleases, in order to fragment the nucleic acids into smaller fragments. These endonucleases may include restriction endonucleases, including type II and type IIs restriction endonucleases as well as other nucleic acid cleaving enzymes, such as nicking endonucleases, and the like. In some cases, fragmentation may not be desired, and full length nucleic acids may be retained within the partitions, or in the case of encapsulated cells or cell contents, fragmentation may be carried out prior to partitioning, e.g., through enzymatic methods, e.g., those described herein, or through mechanical methods, e.g., mechanical, acoustic or other shearing.

Once co-partitioned, and the cells are lysed to release their nucleic acids, the oligonucleotides disposed upon the bead may be used to barcode and amplify fragments of those nucleic acids. A particularly elegant process for use of these barcode oligonucleotides in amplifying and barcoding fragments of sample nucleic acids is described in detail in U.S.

Provisional Patent Application Nos. 61/940,318, filed Feb. 7, 2014, 61/991,018, Filed May 9, 2014, and U.S. patent application Ser. No. 14/316,383, filed Jun. 26, 2014, and previously incorporated by reference. Briefly, in one aspect, the oligonucleotides present on the beads that are co-partitioned with the cells, are released from their beads into the partition with the cell's nucleic acids. The oligonucleotides can include, along with the barcode sequence, a primer sequence at its 5'end. This primer sequence may be a random oligonucleotide sequence intended to randomly prime numerous different regions on the cell's nucleic acids, or it may be a specific primer sequence targeted to prime upstream of a specific targeted region of the cell's genome.

Once released, the primer portion of the oligonucleotide can anneal to a complementary region of the cell's nucleic acid. Extension reaction reagents, e.g., DNA polymerase, nucleoside triphosphates, co-factors (e.g., Mg2+ or Mn2+), that are also co-partitioned with the cells and beads, then extend the primer sequence using the cell's nucleic acid as a template, to produce a complementary fragment to the strand of the cell's nucleic acid to which the primer annealed, which complementary fragment includes the oligonucleotide and its associated barcode sequence. Annealing and extension of multiple primers to different portions of the cell's nucleic acids will result in a large pool of overlapping complementary fragments of the nucleic acid, each possessing its own barcode sequence indicative of the partition in which it was created. In some cases, these complementary fragments may themselves be used as a template primed by the oligonucleotides present in the partition to produce a complement of the complement that again, includes the barcode sequence. In some cases, this replication process is configured such that when the first complement is duplicated, it produces two complementary sequences at or near its termini, to allow formation of a hairpin structure or partial hairpin structure, the reduces the ability of the molecule to be the basis for producing further iterative copies. As described herein, the cell's nucleic acids may include any desired nucleic acids within the cell including, for example, the cell's DNA, e.g., genomic DNA, RNA, e.g., messenger RNA, and the like. For example, in some cases, the methods and systems described herein are used in characterizing expressed mRNA, including, e.g., the presence and quantification of such mRNA, and may include RNA sequencing processes as the characterization process. Alternatively or additionally, the reagents partitioned along with the cells may include reagents for the conversion of mRNA into cDNA, e.g., reverse transcriptase enzymes and reagents, to facilitate sequencing processes where DNA sequencing is employed. In some cases, where the nucleic acids to be characterized comprise RNA, e.g., mRNA, schematic illustration of one example of this is shown in FIG. **3**.

As shown, oligonucleotides that include a barcode sequence are co-partitioned in, e.g., a droplet **302** in an emulsion, along with a sample nucleic acid **304**. As noted elsewhere herein, the oligonucleotides **308** may be provided on a bead **306** that is co-partitioned with the sample nucleic acid **304**, which oligonucleotides are releasable from the bead **306**, as shown in panel A. The oligonucleotides **308** include a barcode sequence **312**, in addition to one or more functional sequences, e.g., sequences **310**, **314** and **316**. For example, oligonucleotide **308** is shown as comprising barcode sequence **312**, as well as sequence **310** that may function as an attachment or immobilization sequence for a given sequencing system, e.g., a P5 sequence used for attachment in flow cells of an Illumina Hiseq® or Miseq® system. As shown, the oligonucleotides also include a primer sequence **316**, which may include a random or targeted N-mer for priming replication of portions of the sample nucleic acid **304**. Also included within oligonucleotide **308** is a sequence **314** which may provide a sequencing priming region, such as a "read1" or R1 priming region, that is used to prime polymerase mediated, template directed sequencing by synthesis reactions in sequencing systems. As will be appreciated, the functional sequences may be selected to be compatible with a variety of different sequencing systems, e.g., 454 Sequencing, Ion Torrent Proton or PGM, Illumina X10, etc., and the requirements thereof. In many cases, the barcode sequence **312**, immobilization sequence **310** and R1 sequence **314** may be common to all of the oligonucleotides attached to a given bead. The primer sequence **316** may vary for random N-mer primers, or may be common to the oligonucleotides on a given bead for certain targeted applications.

As will be appreciated, in some cases, the functional sequences may include primer sequences useful for RNA-seq applications. For example, in some cases, the oligonucleotides may include poly-T primers for priming reverse transcription of RNA for RNA-seq. In still other cases, oligonucleotides in a given partition, e.g., included on an individual bead, may include multiple types of primer sequences in addition to the common barcode sequences, such as both DNA-sequencing and RNA sequencing primers, e.g., poly-T primer sequences included within the oligonucleotides coupled to the bead. In such cases, a single partitioned cell may be both subjected to DNA and RNA sequencing processes.

Based upon the presence of primer sequence **316**, the oligonucleotides can prime the sample nucleic acid as shown in panel B, which allows for extension of the oligonucleotides **308** and **308***a* using polymerase enzymes and other extension reagents also co-partitioned with the bead **306** and sample nucleic acid **304**. As shown in panel C, following extension of the oligonucleotides that, for random N-mer primers, would anneal to multiple different regions of the sample nucleic acid **304**; multiple overlapping complements or fragments of the nucleic acid are created, e.g., fragments **318** and **320**. Although including sequence portions that are complementary to portions of sample nucleic acid, e.g., sequences **322** and **324**, these constructs are generally referred to herein as comprising fragments of the sample nucleic acid **304**, having the attached barcode sequences.

The barcoded nucleic acid fragments may then be subjected to characterization, e.g., through sequence analysis, or they may be further amplified in the process, as shown in panel D. For example, additional oligonucleotides, e.g., oligonucleotide **308***b*, also released from bead **306**, may prime the fragments **318** and **320**. This shown in for fragment **318**. In particular, again, based upon the presence of the random N-mer primer **316***b* in oligonucleotide **308***b* (which in many cases can be different from other random N-mers in a given partition, e.g., primer sequence **316**), the oligonucleotide anneals with the fragment **318**, and is extended to create a complement **326** to at least a portion of fragment **318** which includes sequence **328**, that comprises a duplicate of a portion of the sample nucleic acid sequence. Extension of the oligonucleotide **308***b* continues until it has replicated through the oligonucleotide portion **308** of fragment **318**. As noted elsewhere herein, and as illustrated in panel D, the oligonucleotides may be configured to prompt a stop in the replication by the polymerase at a desired point, e.g., after replicating through sequences **316** and **314** of oligonucleotide **308** that is included within fragment **318**. As

described herein, this may be accomplished by different methods, including, for example, the incorporation of different nucleotides and/or nucleotide analogues that are not capable of being processed by the polymerase enzyme used. For example, this may include the inclusion of uracil containing nucleotides within the sequence region **312** to prevent a non-uracil tolerant polymerase to cease replication of that region. As a result a fragment **326** is created that includes the full-length oligonucleotide **308***b* at one end, including the barcode sequence **312**, the attachment sequence **310**, the R1 primer region **314**, and the random N-mer sequence **316***b*. At the other end of the sequence may be included the complement **316'** to the random N-mer of the first oligonucleotide **308**, as well as a complement to all or a portion of the R1 sequence, shown as sequence **314'**. The R1 sequence **314** and its complement **314'** are then able to hybridize together to form a partial hairpin structure **328**. As will be appreciated because the random N-mers differ among different oligonucleotides, these sequences and their complements would not be expected to participate in hairpin formation, e.g., sequence **316'**, which is the complement to random N-mer **316**, would not be expected to be complementary to random N-mer sequence **316***b*. This would not be the case for other applications, e.g., targeted primers, where the N-mers would be common among oligonucleotides within a given partition.

By forming these partial hairpin structures, it allows for the removal of first level duplicates of the sample sequence from further replication, e.g., preventing iterative copying of copies. The partial hairpin structure also provides a useful structure for subsequent processing of the created fragments, e.g., fragment **326**.

In general, the amplification of the cell's nucleic acids is carried out until the barcoded overlapping fragments within the partition constitute at least 1× coverage of the particular portion or all of the cell's genome, at least 2×, at least 3×, at least 4×, at least 5×, at least 10×, at least 20×, at least 40× or more coverage of the genome or its relevant portion of interest. Once the barcoded fragments are produced, they may be directly sequenced on an appropriate sequencing system, e.g., an Illumina Hiseq®, Miseq® or X10 system, or they may be subjected to additional processing, such as further amplification, attachment of other functional sequences, e.g., second sequencing primers, for reverse reads, sample index sequences, and the like.

All of the fragments from multiple different partitions may then be pooled for sequencing on high throughput sequencers as described herein, where the pooled fragments comprise a large number of fragments derived from the nucleic acids of different cells or small cell populations, but where the fragments from the nucleic acids of a given cell will share the same barcode sequence. In particular, because each fragment is coded as to its partition of origin, and consequently its single cell or small population of cells, the sequence of that fragment may be attributed back to that cell or those cells based upon the presence of the barcode, which will also aid in applying the various sequence fragments from multiple partitions to assembly of individual genomes for different cells. This is schematically illustrated in FIG. **4**. As shown in one example, a first nucleic acid **404** from a first cell **400**, and a second nucleic acid **406** from a second cell **402** are each partitioned along with their own sets of barcode oligonucleotides as described above. The nucleic acids may comprise a chromosome, entire genome or other large nucleic acid from the cells.

Within each partition, each cell's nucleic acids **404** and **406** is then processed to separately provide overlapping set of second fragments of the first fragment), e.g., second fragment sets **408** and **410**. This processing also provides the second fragments with a barcode sequence that is the same for each of the second fragments derived from a particular first fragment. As shown, the barcode sequence for second fragment set **408** is denoted by "1" while the barcode sequence for fragment set **410** is denoted by "2". A diverse library of barcodes may be used to differentially barcode large numbers of different fragment sets. However, it is not necessary for every second fragment set from a different first fragment to be barcoded with different barcode sequences. In fact, in many cases, multiple different first fragments may be processed concurrently to include the same barcode sequence. Diverse barcode libraries are described in detail elsewhere herein.

The barcoded fragments, e.g., from fragment sets **408** and **410**, may then be pooled for sequencing using, for example, sequence by synthesis technologies available from Illumina or Ion Torrent division of Thermo-Fisher, Inc. Once sequenced, the sequence reads **412** can be attributed to their respective fragment set, e.g., as shown in aggregated reads **414** and **416**, at least in part based upon the included barcodes, and in some cases, in part based upon the sequence of the fragment itself. The attributed sequence reads for each fragment set are then assembled to provide the assembled sequence for each cell's nucleic acids, e.g., sequences **418** and **420**, which in turn, may be attributed to individual cells, e.g., cells **400** and **402**.

While described in terms of analyzing the genetic material present within cells, the methods and systems described herein may have much broader applicability, including the ability to characterize other aspects of individual cells or cell populations, by allowing for the allocation of reagents to individual cells, and providing for the attributable analysis or characterization of those cells in response to those reagents. These methods and systems are particularly valuable in being able to characterize cells for, e.g., research, diagnostic, pathogen identification, and many other purposes. By way of example, a wide range of different cell surface features, e.g., cell surface proteins like cluster of differentiation or CD proteins, have significant diagnostic relevance in characterization of diseases like cancer.

In one particularly useful application, the methods and systems described herein may be used to characterize cell features, such as cell surface features, e.g., proteins, receptors, etc. In particular, the methods described herein may be used to attach reporter molecules to these cell features, that when partitioned as described above, may be barcoded and analyzed, e.g., using DNA sequencing technologies, to ascertain the presence, and in some cases, relative abundance or quantity of such cell features within an individual cell or population of cells.

In a particular example, a library of potential cell binding ligands, e.g., antibodies, antibody fragments, cell surface receptor binding molecules, or the like, may be provided associated with a first set of nucleic acid reporter molecules, e.g., where a different reporter oligonucleotide sequence is associated with a specific ligand, and therefore capable of binding to a specific cell surface feature. In some aspects, different members of the library may be characterized by the presence of a different oligonucleotide sequence label, e.g., an antibody to a first type of cell surface protein or receptor would have associated with it a first known reporter oligonucleotide sequence, while an antibody to a second receptor protein would have a different known reporter oligonucleotide sequence associated with it. Prior to co-partitioning, the cells would be incubated with the library of ligands, that

may represent antibodies to a broad panel of different cell surface features, e.g., receptors, proteins, etc., and which include their associated reporter oligonucleotides. Unbound ligands are washed from the cells, and the cells are then co-partitioned along with the barcode oligonucleotides described above. As a result, the partitions will include the cell or cells, as well as the bound ligands and their known, associated reporter oligonucleotides.

Without the need for lysing the cells within the partitions, one could then subject the reporter oligonucleotides to the barcoding operations described above for cellular nucleic acids, to produce barcoded, reporter oligonucleotides, where the presence of the reporter oligonucleotides can be indicative of the presence of the particular cell surface feature, and the barcode sequence will allow the attribution of the range of different cell surface features to a given individual cell or population of cells based upon the barcode sequence that was co-partitioned with that cell or population of cells. As a result, one may generate a cell-by-cell profile of the cell surface features within a broader population of cells. This aspect of the methods and systems described herein, is described in greater detail below.

This example is schematically illustrated in FIG. **5**. As shown, a population of cells, represented by cells **502** and **504** are incubated with a library of cell surface associated reagents, e.g., antibodies, cell surface binding proteins, ligands or the like, where each different type of binding group includes an associated nucleic acid reporter molecule associated with it, shown as ligands and associated reporter molecules **506**, **508**, **510** and **512** (with the reporter molecules being indicated by the differently shaded circles). Where the cell expresses the surface features that are bound by the library, the ligands and their associated reporter molecules can become associated or coupled with the cell surface. Individual cells are then partitioned into separate partitions, e.g., droplets **514** and **516**, along with their associated ligand/reporter molecules, as well as an individual barcode oligonucleotide bead as described elsewhere herein, e.g., beads **522** and **524**, respectively. As with other examples described herein, the barcoded oligonucleotides are released from the beads and used to attach the barcode sequence the reporter molecules present within each partition with a barcode that is common to a given partition, but which varies widely among different partitions. For example, as shown in FIG. **5**, the reporter molecules that associate with cell **502** in partition **514** are barcoded with barcode sequence **518**, while the reporter molecules associated with cell **504** in partition **516** are barcoded with barcode **520**. As a result, one is provided with a library of oligonucleotides that reflects the surface ligands of the cell, as reflected by the reporter molecule, but which is substantially attributable to an individual cell by virtue of a common barcode sequence, allowing a single cell level profiling of the surface characteristics of the cell. As will be appreciated, this process is not limited to cell surface receptors but may be used to identify the presence of a wide variety of specific cell structures, chemistries or other characteristics.

III. BARCODING

Downstream applications, for example DNA sequencing, may rely on the barcodes to identify the origin of a sequence and, for example, to assemble a larger sequence from sequenced fragments. Therefore, it may be desirable to add barcodes to the polynucleotide fragments generated by the methods described herein. Barcodes may be of a variety of different formats, including polynucleotide barcodes. Depending upon the specific application, barcodes may be attached to polynucleotide fragments in a reversible or irreversible manner. Barcodes may also allow for identification and/or quantification of individual polynucleotide fragments during sequencing.

Barcodes may be loaded into partitions so that one or more barcodes are introduced into a particular partition. Each partition may contain a different set of barcodes. This may be accomplished by directly dispensing the barcodes into the partitions, enveloping the barcodes (e.g., in a droplet of an emulsion), or by placing the barcodes within a container that is placed in a partition (e.g., a microcapsule).

For example, a population of microcapsules may be prepared such that a first microcapsule in the population comprises multiple copies of identical barcodes (e.g., polynucleotide bar codes, etc.) and a second microcapsule in the population comprises multiple copies of a barcode that differs from the barcode within the first microcapsule. In some cases, the population of microcapsules may comprise multiple microcapsules (e.g., greater than about 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25, 30, 35, 40, 45, 50, 100, 500, 1000, 5000, 10000, 100000, 1000000, 10000000, 100000000, or 1000000000 microcapsules), each containing multiple copies of a barcode that differs from that contained in the other microcapsules. In some cases, the population may comprise greater than about 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25, 30, 35, 40, 45, 50, 100, 500, 1000, 5000, 10000, 100000, 1000000, 10000000, 100000000, or 1000000000 microcapsules with identical sets of barcodes. In some cases, the population may comprise greater than about 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25, 30, 35, 40, 45, 50, 100, 500, 1000, 5000, 10000, 100000, 1000000, 10000000, 100000000, or 1000000000 microcapsules, wherein the microcapsules each comprise a different combination of barcodes. For example, in some cases the different combinations overlap, such that a first microcapsule may comprise, e.g., barcodes A, B, and C, while a second microcapsule may comprise barcodes A, B, and D. In another example, the different combinations do not overlap, such that a first microcapsule may comprise, e.g., barcodes A, B, and C, while a second microcapsule may comprise barcodes D, E, and F. The use of microcapsules is, of course, optional. All of the combinations described above, and throughout this disclosure, may also be generated by dispending barcodes (and other reagents) directly into partitions (e.g., microwells).

The barcodes may be loaded into the partitions at an expected or predicted ratio of barcodes per species to be barcoded (e.g., polynucleotide fragment, strand of polynucleotide, cell, etc.). In some cases, the barcodes are loaded into partitions such that more than about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 20, 50, 100, 500, 1000, 5000, 10000, or 200000 barcodes are loaded per species. In some cases, the barcodes are loaded in the partitions so that less than about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 20, 50, 100, 500, 1000, 5000, 10000, or 200000 barcodes are loaded per species. In some cases, the average number of barcodes loaded per species is less than, or greater than, about 0.0001, 0.001, 0.01, 0.1, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 20, 50, 100, 500, 1000, 5000, 10000, or 200000 barcodes per species.

When more than one barcode is present per polynucleotide fragment, such barcodes may be copies of the same barcode, or multiple different barcodes. For example, the attachment process may be designed to attach multiple identical barcodes to a single polynucleotide fragment, or multiple different barcodes to the polynucleotide fragment.

A microcapsule may be any of a number of sizes or shapes. In some cases, the shape of the microcapsule may be

spherical, ellipsoidal, cylindrical, hexagonal or any other symmetrical or non-symmetrical shape. Any cross-section of the microcapsule may also be of any appropriate shape, include but not limited to: circular, oblong, square, rectangular, hexagonal, or other symmetrical or non-symmetrical shape. In some cases, the microcapsule may be of a specific shape that complements an opening (e.g., surface of a microwell) of the device. For example, the microcapsule may be spherical and the opening of a microwell of the device may be circular.

The microcapsules may be of uniform size (e.g., all of the microcapsules are the same size) or heterogeneous size (e.g., some of the microcapsules are of different sizes). A dimension (e.g., diameter, cross-section, side, etc.) of a microcapsule may be at least about 0.001 μm, 0.01 μm, 0.1 μm, 0.5 μm, 1 μm, 5 μm, 10 μm, 50 μm, 100 μm, 200 μm, 300 μm, 400 μm, 500 μm, 600 μm, 700 μm, 800 μm, 900 μm or 1 nm. In some cases, the microcapsule comprises a microwell that is at most about 0.001 μm, 0.01 μm, 0.1 μm, 0.5 μm, 1 μm, 5 μm, 10 μm, 50 μm, 100 μm, 200 μm, 300 μm, 400 μm, 500 μm, 600 μm, 700 μm, 800 μm, 900 μm or 1 nm.

In some cases, microcapsules are of a size and/or shape so as to allow a limited number of microcapsules to be deposited in individual partitions (e.g., microwells, droplets) of the microcapsule array. Microcapsules may have a specific size and/or shape such that exactly or no more than 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10 capsules fit into an individual microwell; in some cases, on average 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10 capsules fit into an individual microwell. In still further cases, at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 100, 500, or 1000 capsules fit into an individual microwell.

The methods provided herein may comprise loading a partition (e.g., a microwell, droplet of an emulsion) with the reagents necessary for the attachment of barcodes to polynucleotide fragments. In the case of ligation reactions, reagents including restriction enzymes, ligase enzymes, buffers, adapters, barcodes and the like may be loaded into a partition. In the case barcoding by amplification, reagents including primers, DNA polymerases, DNTPs, buffers, barcodes and the like may be loaded into a partition. As described throughout this disclosure, these reagents may be loaded directly into the partition, or via a container such as a microcapsule. If the reagents are not disposed within a container, they may be loaded into a partition (e.g., a microwell) which may then be sealed with a wax or oil until the reagents are used.

Barcodes may be ligated to a polynucleotide fragment using sticky or blunt ends. Barcoded polynucleotide fragments may also be generated by amplifying a polynucleotide fragment with primers comprising barcodes.

Barcodes may be assembled combinatorially, from smaller components designed to assemble in a modular format. For example, three modules, 1A, 1B, and 1C may be combinatorially assembled to produce barcode 1ABC. Such combinatorial assembly may significantly reduce the cost of synthesizing a plurality of barcodes. For example, a combinatorial system consisting of 3 A modules, 3 B modules, and 3 C modules may generate 3*3*3=27 possible barcode sequences from only 9 modules.

Barcoding and beads of the present disclosure may be performed and used as described in, for example, WO2014/028537 and WO 2014/124338, each of which is entirely incorporated herein by reference.

IV. APPLICATIONS OF SINGLE CELL ANALYSIS

There are a wide variety of different applications of the single cell processing and analysis methods and systems described herein, including analysis of specific individual ells, analysis of different cell types within populations of differing cell types, analysis and characterization of large populations of cells for environmental, human health, epidemiological forensic, or any of a wide variety of different applications.

A particularly valuable application of the single cell analysis processes described herein is in the sequencing and characterization of cancer cells. In particular, conventional analytical techniques, including the ensemble sequencing processes alluded to above, are not highly adept at picking small variations in genomic make-up of cancer cells, particularly where those exist in a sea of normal tissue cells. Further, even as between tumor cells, wide variations can exist and can be masked by the ensemble approaches to sequencing (See, e.g., Patel, et al., Single-cell RNA-seq highlights intratumoral heterogeneity in primary glioblastoma, Science DOI: 10.1126/science.1254257 (Published online Jun. 12, 2014). Cancer cells may be derived from solid tumors, hematological malignancies, cell lines, or obtained as circulating tumor cells, and subjected to the partitioning processes described above. Upon analysis, one can identify individual cell sequences as deriving from a single cell or small group of cells, and distinguish those over normal tissue cell sequences. Further, as described in U.S. Provisional Patent Application No. 62/017,808, filed Jun. 26, 2014, the full disclosures of which is hereby incorporated herein by reference in its entirety for all purposes, one may also obtain phased sequence information from each cell, allowing clearer characterization of the haplotype variants within a cancer cell. The single cell analysis approach is particularly useful for systems and methods involving low quantities of input nucleic acids, as described in U.S. Provisional Patent Application No. 62/017,580, filed Jun. 26, 2014, the full disclosures of which is hereby incorporated herein by reference in its entirety for all purposes.

As with cancer cell analysis, the analysis and diagnosis of fetal health or abnormality through the analysis of fetal cells is a difficult task using conventional techniques. In particular, in the absence of relatively invasive procedures, such as amniocentesis obtaining fetal cell samples can employ harvesting those cells from the maternal circulation. As will be appreciated, such circulating fetal cells make up an extremely small fraction of the overall cellular population of that circulation. As a result complex analyses are performed in order to characterize what of the obtained data is likely derived from fetal cells as opposed to maternal cells. By employing the single cell characterization methods and systems described herein, however, one can attribute genetic make up to individual cells, and categorize those cells as maternal or fetal based upon their respective genetic make-up. Further, the genetic sequence of fetal cells may be used to identify any of a number of genetic disorders, including, e.g., aneuploidy such as Down syndrome, Edwards syndrome, and Patau syndrome.

The ability to characterize individual cells from larger diverse populations of cells is also of significant value in both environmental testing as well as in forensic analysis, where samples may, by their nature, be made up of diverse populations of cells and other material that "contaminate" the sample, relative to the cells for which the sample is being tested, e.g., environmental indicator organisms, toxic organisms, and the like for, e.g., environmental and food safety testing, victim and/or perpetrator cells in forensic analysis for sexual assault, and other violent crimes, and the like.

Additional useful applications of the above described single cell sequencing and characterization processes are in

the field of neuroscience research and diagnosis. In particular, neural cells can include long interspersed nuclear elements (LINEs), or 'jumping' genes that can move around the genome, which cause each neuron to differ from its neighbor cells. Research has shown that the number of LINEs in human brain exceeds that of other tissues, e.g., heart and liver tissue, with between 80 and 300 unique insertions (See, e.g., Coufal, N. G. et al. *Nature* 460, 1127-1131 (2009)). These differences have been postulated as being related to a person's susceptibility to neuro-logical disorders (see, e.g., Muotri, A. R. et al. *Nature* 468, 443-446 (2010)), or provide the brain with a diversity with which to respond to challenges. As such, the methods described herein may be used in the sequencing and characterization of individual neural cells.

The single cell analysis methods described herein are also useful in the analysis of gene expression, as noted above, both in terms of identification of RNA transcripts and their quantitation. In particular, using the single cell level analysis methods described herein, one can isolate and analyze the RNA transcripts present in individual cells, populations of cells, or subsets of populations of cells. In particular, in some cases, the barcode oligonucleotides may be configured to prime, replicate and consequently yield barcoded fragments of RNA from individual cells. For example, in some cases, the barcode oligonucleotides may include mRNA specific priming sequences, e.g., poly-T primer segments that allow priming and replication of mRNA in a reverse transcription reaction or other targeted priming sequences. Alternatively or additionally, random RNA priming may be carried out using random N-mer primer segments of the barcode oligonucleotides.

FIG. **6** provides a schematic of one example method for RNA expression analysis in individual cells using the methods described herein. As shown, at operation **602** a cell containing sample is sorted for viable cells, which are quantified and diluted for subsequent partitioning. At operation **604**, the individual cells separately co-partitioned with gel beads bearing the barcoding oligonucleotides as described herein. The cells are lysed and the barcoded oligonucleotides released into the partitions at operation **606**, where they interact with and hybridize to the mRNA at operation **608**, e.g., by virtue of a poly-T primer sequence, which is complementary to the poly-A tail of the mRNA. Using the poly-T barcode oligonucleotide as a priming sequence, a reverse transcription reaction is carried out at operation **610** to synthesize a cDNA transcript of the mRNA that includes the barcode sequence. The barcoded cDNA transcripts are then subjected to additional amplification at operation **612**, e.g., using a PCR process, purification at operation **614**, before they are placed on a nucleic acid sequencing system for determination of the cDNA sequence and its associated barcode sequence(s). In some cases, as shown, operations **602** through **608** can occur while the reagents remain in their original droplet or partition, while operations **612** through **616** can occur in bulk (e.g., outside of the partition). In the case where a partition is a droplet in an emulsion, the emulsion can be broken and the contents of the droplet pooled in order to complete operations **612** through **616**. In some cases, barcode oligonucleotides may be digested with exonucleases after the emulsion is broken. Exonuclease activity can be inhibited by ethylenediaminetetraacetic acid (EDTA) following primer digestion. In some cases, operation **610** may be performed either within the partitions based upon co-partitioning of the reverse transcription mixture, e.g., reverse transcriptase and associated reagents, or it may be performed in bulk.

As noted elsewhere herein, the structure of the barcode oligonucleotides may include a number of sequence elements in addition to the oligonucleotide barcode sequence. One example of a barcode oligonucleotide for use in RNA analysis as described above is shown in FIG. **7**. As shown, the overall oligonucleotide **702** is coupled to a bead **704** by a releasable linkage **706**, such as a disulfide linker. The oligonucleotide may include functional sequences that are used in subsequent processing, such as functional sequence **708**, which may include one or more of a sequencer specific flow cell attachment sequence, e.g., a P5 sequence for Illumina sequencing systems, as well as sequencing primer sequences, e.g., a R1 primer for Illumina sequencing systems. A barcode sequence **710** is included within the structure for use in barcoding the sample RNA. An mRNA specific priming sequence, such as poly-T sequence **712** is also included in the oligonucleotide structure. An anchoring sequence segment **714** may be included to ensure that the poly-T sequence hybridizes at the sequence end of the mRNA. This anchoring sequence can include a random short sequence of nucleotides, e.g., 1-mer, 2-mer, 3-mer or longer sequence, which will ensure that the poly-T segment is more likely to hybridize at the sequence end of the poly-A tail of the mRNA. An additional sequence segment **716** may be provided within the oligonucleotide sequence. In some cases, this additional sequence provides a unique molecular sequence segment, e.g., as a random sequence (e.g., such as a random N-mer sequence) that varies across individual oligonucleotides coupled to a single bead, whereas barcode sequence **710** can be constant among oligonucleotides tethered to an individual bead. This unique sequence serves to provide a unique identifier of the starting mRNA molecule that was captured, in order to allow quantitation of the number of original expressed RNA. As will be appreciated, although shown as a single oligonucleotide tethered to the surface of a bead, individual bead can include tens to hundreds of thousands or even millions of individual oligonucleotide molecules, where, as noted, the barcode segment can be constant or relatively constant for a given bead, but where the variable or unique sequence segment will vary across an individual bead. This unique molecular sequence segment may include from 5 to about 8 or more nucleotides within the sequence of the oligonucleotides. In some cases, the unique molecular sequence segment can be 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19 or 20 nucleotides in length or longer. In some cases, the unique molecular sequence segment can be at least 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19 or 20 nucleotides in length or longer. In some cases, the unique molecular sequence segment can be at most 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19 or 20 nucleotides in length or shorter.

In operation, and with reference to FIGS. **6** and **7**, a cell is co-partitioned along with a barcode bearing bead and lysed while the barcoded oligonucleotides are released from the bead. The poly-T portion of the released barcode oligonucleotide then hybridizes to the poly-A tail of the mRNA. The poly-T segment then primes the reverse transcription of the mRNA to produce a cDNA transcript of the mRNA, but which includes each of the sequence segments **708-716** of the barcode oligonucleotide. Again, because the oligonucleotide **702** includes an anchoring sequence **714**, it will more likely hybridize to and prime reverse transcription at the sequence end of the poly-A tail of the mRNA. Within any given partition, all of the cDNA transcripts of the individual mRNA molecules will include a common barcode sequence segment **710**. However, by including the unique random N-mer sequence, the transcripts made from different mRNA

molecules within a given partition will vary at this unique sequence. This provides a quantitation feature that can be identifiable even following any subsequent amplification of the contents of a given partition, e.g., the number of unique segments associated with a common barcode can be indicative of the quantity of mRNA originating from a single partition, and thus, a single cell. As noted above, the transcripts are then amplified, cleaned up and sequenced to identify the sequence of the cDNA transcript of the mRNA, as well as to sequence the barcode segment and the unique sequence segment.

As noted elsewhere herein, while a poly-T primer sequence is described, other targeted or random priming sequences may also be used in priming the reverse transcription reaction. Likewise, although described as releasing the barcoded oligonucleotides into the partition along with the contents of the lysed cells, it will be appreciated that in some cases, the gel bead bound oligonucleotides may be used to hybridize ad capture the mRNA on the solid phase of the gel beads, in order to facilitate the separation of the RNA from other cell contents.

An additional example of a barcode oligonucleotide for use in RNA analysis, including messenger RNA (mRNA, including mRNA obtained from a cell) analysis, is shown in FIG. **9**A. As shown, the overall oligonucleotide **902** can be coupled to a bead **904** by a releasable linkage **906**, such as a disulfide linker. The oligonucleotide may include functional sequences that are used in subsequent processing, such as functional sequence **908**, which may include a sequencer specific flow cell attachment sequence, e.g., a P5 sequence for Illumina sequencing systems, as well as functional sequence **910**, which may include sequencing primer sequences, e.g., a R1 primer binding site for Illumina sequencing systems. A barcode sequence **912** is included within the structure for use in barcoding the sample RNA. An RNA specific (e.g., mRNA specific) priming sequence, such as poly-T sequence **914** is also included in the oligonucleotide structure. An anchoring sequence segment (not shown) may be included to ensure that the poly-T sequence hybridizes at the sequence end of the mRNA. An additional sequence segment **916** may be provided within the oligonucleotide sequence. This additional sequence can provide a unique molecular sequence segment, e.g., as a random N-mer sequence that varies across individual oligonucleotides coupled to a single bead, whereas barcode sequence **912** can be constant among oligonucleotides tethered to an individual bead. As described elsewhere herein, this unique sequence can serve to provide a unique identifier of the starting mRNA molecule that was captured, in order to allow quantitation of the number of original expressed RNA, e.g., mRNA counting. As will be appreciated, although shown as a single oligonucleotide tethered to the surface of a bead, individual beads can include tens to hundreds of thousands or even millions of individual oligonucleotide molecules, where, as noted, the barcode segment can be constant or relatively constant for a given bead, but where the variable or unique sequence segment will vary across an individual bead.

In an example method of cellular RNA (e.g., mRNA) analysis and in reference to FIG. **9**A, a cell is co-partitioned along with a barcode bearing bead, switch oligo **924**, and other reagents such as reverse transcriptase, a reducing agent and dNTPs into a partition (e.g., a droplet in an emulsion). In operation **950**, the cell is lysed while the barcoded oligonucleotides **902** are released from the bead (e.g., via the action of the reducing agent) and the poly-T segment **914** of the released barcode oligonucleotide then hybridizes to the poly-A tail of mRNA **920** that is released from the cell. Next, in operation **952** the poly-T segment **914** is extended in a reverse transcription reaction using the mRNA as a template to produce a cDNA transcript **922** complementary to the mRNA and also includes each of the sequence segments **908**, **912**, **910**, **916** and **914** of the barcode oligonucleotide. Terminal transferase activity of the reverse transcriptase can add additional bases to the cDNA transcript (e.g., polyC). The switch oligo **924** may then hybridize with the additional bases added to the cDNA transcript and facilitate template switching. A sequence complementary to the switch oligo sequence can then be incorporated into the cDNA transcript **922** via extension of the cDNA transcript **922** using the switch oligo **924** as a template. Within any given partition, all of the cDNA transcripts of the individual mRNA molecules will include a common barcode sequence segment **912**. However, by including the unique random N-mer sequence **916**, the transcripts made from different mRNA molecules within a given partition will vary at this unique sequence. As described elsewhere herein, this provides a quantitation feature that can be identifiable even following any subsequent amplification of the contents of a given partition, e.g., the number of unique segments associated with a common barcode can be indicative of the quantity of mRNA originating from a single partition, and thus, a single cell. Following operation **952**, the cDNA transcript **922** is then amplified with primers **926** (e.g., PCR primers) in operation **954**. Next, the amplified product is then purified (e.g., via solid phase reversible immobilization (SPRI)) in operation **956**. At operation **958**, the amplified product is then sheared, ligated to additional functional sequences, and further amplified (e.g., via PCR). The functional sequences may include a sequencer specific flow cell attachment sequence **930**, e.g., a P7 sequence for Illumina sequencing systems, as well as functional sequence **928**, which may include a sequencing primer binding site, e.g., for a R2 primer for Illumina sequencing systems, as well as functional sequence **932**, which may include a sample index, e.g., an i7 sample index sequence for Illumina sequencing systems. In some cases, operations **950** and **952** can occur in the partition, while operations **954**, **956** and **958** can occur in bulk solution (e.g., in a pooled mixture outside of the partition). In the case where a partition is a droplet in an emulsion, the emulsion can be broken and the contents of the droplet pooled in order to complete operations **954**, **956** and **958**. In some cases, operation **954** may be completed in the partition. In some cases, barcode oligonucleotides may be digested with exonucleases after the emulsion is broken. Exonuclease activity can be inhibited by ethylenediaminetetraacetic acid (EDTA) following primer digestion. Although described in terms of specific sequence references used for certain sequencing systems, e.g., Illumina systems, it will be understood that the reference to these sequences is for illustration purposes only, and the methods described herein may be configured for use with other sequencing systems incorporating specific priming, attachment, index, and other operational sequences used in those systems, e.g., systems available from Ion Torrent, Oxford Nanopore, Genia, Pacific Biosciences, Complete Genomics, and the like.

In an alternative example of a barcode oligonucleotide for use in RNA (e.g., cellular RNA) analysis as shown in FIG. **9**A, functional sequence **908** may be a P7 sequence and functional sequence **910** may be a R2 primer binding site. Moreover, the functional sequence **930** may be a P5 sequence, functional sequence **928** may be a R1 primer binding site, and functional sequence **932** may be an i5

sample index sequence for Illumina sequencing systems. The configuration of the constructs generated by such a barcode oligonucleotide can help minimize (or avoid) sequencing of the poly-T sequence during sequencing.

Shown in FIG. **9**B is another example method for RNA analysis, including cellular mRNA analysis. In this method, the switch oligo **924** is co-partitioned with the individual cell and barcoded bead along with reagents such as reverse transcriptase, a reducing agent and dNTPs into a partition (e.g., a droplet in an emulsion). The switch oligo **924** may be labeled with an additional tag **934**, e.g. biotin. In operation **951**, the cell is lysed while the barcoded oligonucleotides **902** (e.g., as shown in FIG. **9**A) are released from the bead (e.g., via the action of the reducing agent). In some cases, sequence **908** is a P7 sequence and sequence **910** is a R2 primer binding site. In other cases, sequence **908** is a P5 sequence and sequence **910** is a R1 primer binding site. Next, the poly-T segment **914** of the released barcode oligonucleotide hybridizes to the poly-A tail of mRNA **920** that is released from the cell. In operation **953**, the poly-T segment **914** is then extended in a reverse transcription reaction using the mRNA as a template to produce a cDNA transcript **922** complementary to the mRNA and also includes each of the sequence segments **908**, **912**, **910**, **916** and **914** of the barcode oligonucleotide. Terminal transferase activity of the reverse transcriptase can add additional bases to the cDNA transcript (e.g., polyC). The switch oligo **924** may then hybridize with the cDNA transcript and facilitate template switching. A sequence complementary to the switch oligo sequence can then be incorporated into the cDNA transcript **922** via extension of the cDNA transcript **922** using the switch oligo **924** as a template. Next, an isolation operation **960** can be used to isolate the cDNA transcript **922** from the reagents and oligonucleotides in the partition. The additional tag **934**, e.g. biotin, can be contacted with an interacting tag **936**, e.g., streptavidin, which may be attached to a magnetic bead **938**. At operation **960** the cDNA can be isolated with a pull-down operation (e.g., via magnetic separation, centrifugation) before amplification (e.g., via PCR) in operation **955**, followed by purification (e.g., via solid phase reversible immobilization (SPRI)) in operation **957** and further processing (shearing, ligation of sequences **928**, **932** and **930** and subsequent amplification (e.g., via PCR)) in operation **959**. In some cases where sequence **908** is a P7 sequence and sequence **910** is a R2 primer binding site, sequence **930** is a P5 sequence and sequence **928** is a R1 primer binding site and sequence **932** is an i5 sample index sequence. In some cases where sequence **908** is a P5 sequence and sequence **910** is a R1 primer binding site, sequence **930** is a P7 sequence and sequence **928** is a R2 primer binding site and sequence **932** is an i7 sample index sequence. In some cases, as shown, operations **951** and **953** can occur in the partition, while operations **960**, **955**, **957** and **959** can occur in bulk solution (e.g., in a pooled mixture outside of the partition). In the case where a partition is a droplet in an emulsion, the emulsion can be broken and the contents of the droplet pooled in order to complete operation **960**. The operations **955**, **957**, and **959** can then be carried out following operation **960** after the transcripts are pooled for processing.

Shown in FIG. **9**C is another example method for RNA analysis, including cellular mRNA analysis. In this method, the switch oligo **924** is co-partitioned with the individual cell and barcoded bead along with reagents such as reverse transcriptase, a reducing agent and dNTPs in a partition (e.g., a droplet in an emulsion). In operation **961**, the cell is lysed while the barcoded oligonucleotides **902** (e.g., as shown in FIG. **9**A) are released from the bead (e.g., via the action of the reducing agent). In some cases, sequence **908** is a P7 sequence and sequence **910** is a R2 primer binding site. In other cases, sequence **908** is a P5 sequence and sequence **910** is a R1 primer binding site. Next, the poly-T segment **914** of the released barcode oligonucleotide then hybridizes to the poly-A tail of mRNA **920** that is released from the cell. Next, in operation **963** the poly-T segment **914** is then extended in a reverse transcription reaction using the mRNA as a template to produce a cDNA transcript **922** complementary to the mRNA and also includes each of the sequence segments **908**, **912**, **910**, **916** and **914** of the barcode oligonucleotide. Terminal transferase activity of the reverse transcriptase can add additional bases to the cDNA transcript (e.g., polyC). The switch oligo **924** may then hybridize with the cDNA transcript and facilitate template switching. A sequence complementary to the switch oligo sequence can then be incorporated into the cDNA transcript **922** via extension of the cDNA transcript **922** using the switch oligo **924** as a template. Following operation **961** and operation **963**, mRNA **920** and cDNA transcript **922** are denatured in operation **962**. At operation **964**, a second strand is extended from a primer **940** having an additional tag **942**, e.g. biotin, and hybridized to the cDNA transcript **922**. Also in operation **964**, the biotin labeled second strand can be contacted with an interacting tag **936**, e.g. streptavidin, which may be attached to a magnetic bead **938**. The cDNA can be isolated with a pull-down operation (e.g., via magnetic separation, centrifugation) before amplification (e.g., via polymerase chain reaction (PCR)) in operation **965**, followed by purification (e.g., via solid phase reversible immobilization (SPRI)) in operation **967** and further processing (shearing, ligation of sequences **928**, **932** and **930** and subsequent amplification (e.g., via PCR)) in operation **969**. In some cases where sequence **908** is a P7 sequence and sequence **910** is a R2 primer binding site, sequence **930** is a P5 sequence and sequence **928** is a R1 primer binding site and sequence **932** is an i5 sample index sequence. In some cases where sequence **908** is a P5 sequence and sequence **910** is a R1 primer binding site, sequence **930** is a P7 sequence and sequence **928** is a R2 primer binding site and sequence **932** is an i7 sample index sequence. In some cases, operations **961** and **963** can occur in the partition, while operations **962**, **964**, **965**, **967**, and **969** can occur in bulk (e.g., outside the partition). In the case where a partition is a droplet in an emulsion, the emulsion can be broken and the contents of the droplet pooled in order to complete operations **962**, **964**, **965**, **967** and **969**.

Shown in FIG. **9**D is another example method for RNA analysis, including cellular mRNA analysis. In this method, the switch oligo **924** is co-partitioned with the individual cell and barcoded bead along with reagents such as reverse transcriptase, a reducing agent and dNTPs. In operation **971**, the cell is lysed while the barcoded oligonucleotides **902** (e.g., as shown in FIG. **9**A) are released from the bead (e.g., via the action of the reducing agent). In some cases, sequence **908** is a P7 sequence and sequence **910** is a R2 primer binding site. In other cases, sequence **908** is a P5 sequence and sequence **910** is a R1 primer binding site. Next the poly-T segment **914** of the released barcode oligonucleotide then hybridizes to the poly-A tail of mRNA **920** that is released from the cell. Next in operation **973**, the poly-T segment **914** is then extended in a reverse transcription reaction using the mRNA as a template to produce a cDNA transcript **922** complementary to the mRNA and also includes each of the sequence segments **908**, **912**, **910**, **916** and **914** of the barcode oligonucleotide. Terminal transferase

activity of the reverse transcriptase can add additional bases to the cDNA transcript (e.g., polyC). The switch oligo **924** may then hybridize with the cDNA transcript and facilitate template switching. A sequence complementary to the switch oligo sequence can then be incorporated into the cDNA transcript **922** via extension of the cDNA transcript **922** using the switch oligo **924** as a template. In operation **966**, the mRNA **920**, cDNA transcript **922** and switch oligo **924** can be denatured, and the cDNA transcript **922** can be hybridized with a capture oligonucleotide **944** labeled with an additional tag **946**, e.g. biotin. In this operation, the biotin-labeled capture oligonucleotide **944**, which is hybridized to the cDNA transcript, can be contacted with an interacting tag **936**, e.g. streptavidin, which may be attached to a magnetic bead **938**. Following separation from other species (e.g., excess barcoded oligonucleotides) using a pull-down operation (e.g., via magnetic separation, centrifugation), the cDNA transcript can be amplified (e.g., via PCR) with primers **926** at operation **975**, followed by purification (e.g., via solid phase reversible immobilization (SPRI)) in operation **977** and further processing (shearing, ligation of sequences **928**, **932** and **930** and subsequent amplification (e.g., via PCR)) in operation **979**. In some cases where sequence **908** is a P7 sequence and sequence **910** is a R2 primer binding site, sequence **930** is a P5 sequence and sequence **928** is a R1 primer binding site and sequence **932** is an i5 sample index sequence. In other cases where sequence **908** is a P5 sequence and sequence **910** is a R1 primer binding site, sequence **930** is a P7 sequence and sequence **928** is a R2 primer binding site and sequence **932** is an i7 sample index sequence. In some cases, operations **971** and **973** can occur in the partition, while operations **966**, **975**, **977** (purification), and **979** can occur in bulk (e.g., outside the partition). In the case where a partition is a droplet in an emulsion, the emulsion can be broken and the contents of the droplet pooled in order to complete operations **966**, **975**, **977** and **979**.

Shown in FIG. **9**E is another example method for RNA analysis, including cellular RNA analysis. In this method, an individual cell is co-partitioned along with a barcode bearing bead, a switch oligo **990**, and other reagents such as reverse transcriptase, a reducing agent and dNTPs into a partition (e.g., a droplet in an emulsion). In operation **981**, the cell is lysed while the barcoded oligonucleotides (e.g., **902** as shown in FIG. **9**A) are released from the bead (e.g., via the action of the reducing agent). In some cases, sequence **908** is a P7 sequence and sequence **910** is a R2 primer binding site. In other cases, sequence **908** is a P5 sequence and sequence **910** is a R1 primer binding site. Next, the poly-T segment of the released barcode oligonucleotide then hybridizes to the poly-A tail of mRNA **920** released from the cell. Next at operation **983**, the poly-T segment is then extended in a reverse transcription reaction to produce a cDNA transcript **922** complementary to the mRNA and also includes each of the sequence segments **908**, **912**, **910**, **916** and **914** of the barcode oligonucleotide. Terminal transferase activity of the reverse transcriptase can add additional bases to the cDNA transcript (e.g., polyC). The switch oligo **990** may then hybridize with the cDNA transcript and facilitate template switching. A sequence complementary to the switch oligo sequence and including a T7 promoter sequence, can be incorporated into the cDNA transcript **922**. At operation **968**, a second strand is synthesized and at operation **970** the T7 promoter sequence can be used by T7 polymerase to produce RNA transcripts in in vitro transcription. At operation **985** the RNA transcripts can be purified (e.g., via solid phase reversible immobilization (SPRI)), reverse transcribed to form DNA transcripts, and a second strand can be synthesized for each of the DNA transcripts. In some cases, prior to purification, the RNA transcripts can be contacted with a DNase (e.g., DNAase I) to break down residual DNA. At operation **987** the DNA transcripts are then fragmented and ligated to additional functional sequences, such as sequences **928**, **932** and **930** and, in some cases, further amplified (e.g., via PCR). In some cases where sequence **908** is a P7 sequence and sequence **910** is a R2 primer binding site, sequence **930** is a P5 sequence and sequence **928** is a R1 primer binding site and sequence **932** is an i5 sample index sequence. In some cases where sequence **908** is a P5 sequence and sequence **910** is a R1 primer binding site, sequence **930** is a P7 sequence and sequence **928** is a R2 primer binding site and sequence **932** is an i7 sample index sequence. In some cases, prior to removing a portion of the DNA transcripts, the DNA transcripts can be contacted with an RNase to break down residual RNA. In some cases, operations **981** and **983** can occur in the partition, while operations **968**, **970**, **985** and **987** can occur in bulk (e.g., outside the partition). In the case where a partition is a droplet in an emulsion, the emulsion can be broken and the contents of the droplet pooled in order to complete operations **968**, **970**, **985** and **987**.

Another example of a barcode oligonucleotide for use in RNA analysis, including messenger RNA (mRNA, including mRNA obtained from a cell) analysis is shown in FIG. **10**. As shown, the overall oligonucleotide **1002** is coupled to a bead **1004** by a releasable linkage **1006**, such as a disulfide linker. The oligonucleotide may include functional sequences that are used in subsequent processing, such as functional sequence **1008**, which may include a sequencer specific flow cell attachment sequence, e.g., a P7 sequence, as well as functional sequence **1010**, which may include sequencing primer sequences, e.g., a R2 primer binding site. A barcode sequence **1012** is included within the structure for use in barcoding the sample RNA. An RNA specific (e.g., mRNA specific) priming sequence, such as poly-T sequence **1014** may be included in the oligonucleotide structure. An anchoring sequence segment (not shown) may be included to ensure that the poly-T sequence hybridizes at the sequence end of the mRNA. An additional sequence segment **1016** may be provided within the oligonucleotide sequence. This additional sequence can provide a unique molecular sequence segment, as described elsewhere herein. An additional functional sequence **1020** may be included for in vitro transcription, e.g., a T7 RNA polymerase promoter sequence. As will be appreciated, although shown as a single oligonucleotide tethered to the surface of a bead, individual beads can include tens to hundreds of thousands or even millions of individual oligonucleotide molecules, where, as noted, the barcode segment can be constant or relatively constant for a given bead, but where the variable or unique sequence segment will vary across an individual bead.

In an example method of cellular RNA analysis and in reference to FIG. **10**, a cell is co-partitioned along with a barcode bearing bead, and other reagents such as reverse transcriptase, reducing agent and dNTPs into a partition (e.g., a droplet in an emulsion). In operation **1050**, the cell is lysed while the barcoded oligonucleotides **1002** are released (e.g., via the action of the reducing agent) from the bead, and the poly-T segment **1014** of the released barcode oligonucleotide then hybridizes to the poly-A tail of mRNA **1020**. Next at operation **1052**, the poly-T segment is then extended in a reverse transcription reaction using the mRNA as template to produce a cDNA transcript **1022** of the mRNA and also includes each of the sequence segments **1020**, **1008**,

1012, **1010**, **1016**, and **1014** of the barcode oligonucleotide. Within any given partition, all of the cDNA transcripts of the individual mRNA molecules will include a common barcode sequence segment **1012**. However, by including the unique random N-mer sequence, the transcripts made from different mRNA molecules within a given partition will vary at this unique sequence. As described elsewhere herein, this provides a quantitation feature that can be identifiable even following any subsequent amplification of the contents of a given partition, e.g., the number of unique segments associated with a common barcode can be indicative of the quantity of mRNA originating from a single partition, and thus, a single cell. At operation **1054** a second strand is synthesized and at operation **1056** the T7 promoter sequence can be used by T7 polymerase to produce RNA transcripts in in vitro transcription. At operation **1058** the transcripts are fragmented (e.g., sheared), ligated to additional functional sequences, and reverse transcribed. The functional sequences may include a sequencer specific flow cell attachment sequence **1030**, e.g., a P5 sequence, as well as functional sequence **1028**, which may include sequencing primers, e.g., a R1 primer binding sequence, as well as functional sequence **1032**, which may include a sample index, e.g., an i5 sample index sequence. At operation **1060** the RNA transcripts can be reverse transcribed to DNA, the DNA amplified (e.g., via PCR), and sequenced to identify the sequence of the cDNA transcript of the mRNA, as well as to sequence the barcode segment and the unique sequence segment. In some cases, operations **1050** and **1052** can occur in the partition, while operations **1054**, **1056**, **1058** and **1060** can occur in bulk (e.g., outside the partition). In the case where a partition is a droplet in an emulsion, the emulsion can be broken and the contents of the droplet pooled in order to complete operations **1054**, **1056**, **1058** and **1060**.

In an alternative example of a barcode oligonucleotide for use in RNA (e.g., cellular RNA) analysis as shown in FIG. **10**, functional sequence **1008** may be a P5 sequence and functional sequence **1010** may be a R1 primer binding site. Moreover, the functional sequence **1030** may be a P7 sequence, functional sequence **1028** may be a R2 primer binding site, and functional sequence **1032** may be an i7 sample index sequence.

An additional example of a barcode oligonucleotide for use in RNA analysis, including messenger RNA (mRNA, including mRNA obtained from a cell) analysis is shown in FIG. **11**. As shown, the overall oligonucleotide **1102** is coupled to a bead **1104** by a releasable linkage **1106**, such as a disulfide linker. The oligonucleotide may include functional sequences that are used in subsequent processing, such as functional sequence **1108**, which may include a sequencer specific flow cell attachment sequence, e.g., a P5 sequence, as well as functional sequence **1110**, which may include sequencing primer sequences, e.g., a R1 primer binding site. In some cases, sequence **1108** is a P7 sequence and sequence **1110** is a R2 primer binding site. A barcode sequence **1112** is included within the structure for use in barcoding the sample RNA. An additional sequence segment **1116** may be provided within the oligonucleotide sequence. In some cases, this additional sequence can provide a unique molecular sequence segment, as described elsewhere herein. An additional sequence **1114** may be included to facilitate template switching, e.g., polyG. As will be appreciated, although shown as a single oligonucleotide tethered to the surface of a bead, individual beads can include tens to hundreds of thousands or even millions of individual oligonucleotide molecules, where, as noted, the barcode segment can be constant or relatively constant for a given bead, but where the variable or unique sequence segment will vary across an individual bead.

In an example method of cellular mRNA analysis and in reference to FIG. **11**, a cell is co-partitioned along with a barcode bearing bead, poly-T sequence, and other reagents such as reverse transcriptase, a reducing agent and dNTPs into a partition (e.g., a droplet in an emulsion). In operation **1150**, the cell is lysed while the barcoded oligonucleotides are released from the bead (e.g., via the action of the reducing agent) and the poly-T sequence hybridizes to the poly-A tail of mRNA **1120** released from the cell. Next, in operation **1152**, the poly-T sequence is then extended in a reverse transcription reaction using the mRNA as a template to produce a cDNA transcript **1122** complementary to the mRNA. Terminal transferase activity of the reverse transcriptase can add additional bases to the cDNA transcript (e.g., polyC). The additional bases added to the cDNA transcript, e.g., polyC, can then to hybridize with **1114** of the barcoded oligonucleotide. This can facilitate template switching and a sequence complementary to the barcode oligonucleotide can be incorporated into the cDNA transcript. The transcripts can be further processed (e.g., amplified, portions removed, additional sequences added, etc.) and characterized as described elsewhere herein, e.g., by sequencing. The configuration of the constructs generated by such a method can help minimize (or avoid) sequencing of the poly-T sequence during sequencing.

An additional example of a barcode oligonucleotide for use in RNA analysis, including cellular RNA analysis is shown in FIG. **12**A. As shown, the overall oligonucleotide **1202** is coupled to a bead **1204** by a releasable linkage **1206**, such as a disulfide linker. The oligonucleotide may include functional sequences that are used in subsequent processing, such as functional sequence **1208**, which may include a sequencer specific flow cell attachment sequence, e.g., a P5 sequence, as well as functional sequence **1210**, which may include sequencing primer sequences, e.g., a R1 primer binding site. In some cases, sequence **1208** is a P7 sequence and sequence **1210** is a R2 primer binding site. A barcode sequence **1212** is included within the structure for use in barcoding the sample RNA. An additional sequence segment **1216** may be provided within the oligonucleotide sequence. In some cases, this additional sequence can provide a unique molecular sequence segment, as described elsewhere herein. As will be appreciated, although shown as a single oligonucleotide tethered to the surface of a bead, individual beads can include tens to hundreds of thousands or even millions of individual oligonucleotide molecules, where, as noted, the barcode segment can be constant or relatively constant for a given bead, but where the variable or unique sequence segment will vary across an individual bead. In an example method of cellular RNA analysis using this barcode, a cell is co-partitioned along with a barcode bearing bead and other reagents such as RNA ligase and a reducing agent into a partition (e.g. a droplet in an emulsion). The cell is lysed while the barcoded oligonucleotides are released (e.g., via the action of the reducing agent) from the bead. The barcoded oligonucleotides can then be ligated to the 5' end of mRNA transcripts while in the partitions by RNA ligase. Subsequent operations may include purification (e.g., via solid phase reversible immobilization (SPRI)) and further processing (shearing, ligation of functional sequences, and subsequent amplification (e.g., via PCR)), and these operations may occur in bulk (e.g., outside the partition). In the case where a partition is a droplet in an emulsion, the

emulsion can be broken and the contents of the droplet pooled for the additional operations.

An additional example of a barcode oligonucleotide for use in RNA analysis, including cellular RNA analysis is shown in FIG. **12**B. As shown, the overall oligonucleotide **1222** is coupled to a bead **1224** by a releasable linkage **1226**, such as a disulfide linker. The oligonucleotide may include functional sequences that are used in subsequent processing, such as functional sequence **1228**, which may include a sequencer specific flow cell attachment sequence, e.g., a P5 sequence, as well as functional sequence **1230**, which may include sequencing primer sequences, e.g., a R1 primer binding site. In some cases, sequence **1228** is a P7 sequence and sequence **1230** is a R2 primer binding site. A barcode sequence **1232** is included within the structure for use in barcoding the sample RNA. A priming sequence **1234** (e.g., a random priming sequence) can also be included in the oligonucleotide structure, e.g., a random hexamer. An additional sequence segment **1236** may be provided within the oligonucleotide sequence. In some cases, this additional sequence provides a unique molecular sequence segment, as described elsewhere herein. As will be appreciated, although shown as a single oligonucleotide tethered to the surface of a bead, individual beads can include tens to hundreds of thousands or even millions of individual oligonucleotide molecules, where, as noted, the barcode segment can be constant or relatively constant for a given bead, but where the variable or unique sequence segment will vary across an individual bead. In an example method of cellular mRNA analysis using the barcode oligonucleotide of FIG. **12**B, a cell is co-partitioned along with a barcode bearing bead and additional reagents such as reverse transcriptase, a reducing agent and dNTPs into a partition (e.g., a droplet in an emulsion). The cell is lysed while the barcoded oligonucleotides are released from the bead (e.g., via the action of the reducing agent). In some cases, sequence **1228** is a P7 sequence and sequence **1230** is a R2 primer binding site. In other cases, sequence **1228** is a P5 sequence and sequence **1230** is a R1 primer binding site. The priming sequence **1234** of random hexamers can randomly hybridize cellular mRNA. The random hexamer sequence can then be extended in a reverse transcription reaction using mRNA from the cell as a template to produce a cDNA transcript complementary to the mRNA and also includes each of the sequence segments **1228**, **1232**, **1230**, **1236**, and **1234** of the barcode oligonucleotide. Subsequent operations may include purification (e.g., via solid phase reversible immobilization (SPRI)), further processing (shearing, ligation of functional sequences, and subsequent amplification (e.g., via PCR)), and these operations may occur in bulk (e.g., outside the partition). In the case where a partition is a droplet in an emulsion, the emulsion can be broken and the contents of the droplet pooled for additional operations. Additional reagents that may be co-partitioned along with the barcode bearing bead may include oligonucleotides to block ribosomal RNA (rRNA) and nucleases to digest genomic DNA and cDNA from cells. Alternatively, rRNA removal agents may be applied during additional processing operations. The configuration of the constructs generated by such a method can help minimize (or avoid) sequencing of the poly-T sequence during sequencing.

The single cell analysis methods described herein may also be useful in the analysis of the whole transcriptome. Referring back to the barcode of FIG. **12**B, the priming sequence **1234** may be a random N-mer. In some cases, sequence **1228** is a P7 sequence and sequence **1230** is a R2 primer binding site. In other cases, sequence **1228** is a P5 sequence and sequence **1230** is a R1 primer binding site. In an example method of whole transcriptome analysis using this barcode, the individual cell is co-partitioned along with a barcode bearing bead, poly-T sequence, and other reagents such as reverse transcriptase, polymerase, a reducing agent and dNTPs into a partition (e.g., droplet in an emulsion). In an operation of this method, the cell is lysed while the barcoded oligonucleotides are released from the bead (e.g., via the action of the reducing agent) and the poly-T sequence hybridizes to the poly-A tail of cellular mRNA. In a reverse transcription reaction using the mRNA as template, cDNA transcripts of cellular mRNA can be produced. The RNA can then be degraded with an RNase. The priming sequence **1234** in the barcoded oligonucleotide can then randomly hybridize to the cDNA transcripts. The oligonucleotides can be extended using polymerase enzymes and other extension reagents co-partitioned with the bead and cell similar to as shown in FIG. **3** to generate amplification products (e.g., barcoded fragments), similar to the example amplification product shown in FIG. **3** (panel F). The barcoded nucleic acid fragments may, in some cases subjected to further processing (e.g., amplification, addition of additional sequences, clean up processes, etc. as described elsewhere herein) characterized, e.g., through sequence analysis. In this operation, sequencing signals can come from full length RNA.

Although operations with various barcode designs have been discussed individually, individual beads can include barcode oligonucleotides of various designs for simultaneous use.

In addition to characterizing individual cells or cell subpopulations from larger populations, the processes and systems described herein may also be used to characterize individual cells as a way to provide an overall profile of a cellular, or other organismal population. A variety of applications require the evaluation of the presence and quantification of different cell or organism types within a population of cells, including, for example, microbiome analysis and characterization, environmental testing, food safety testing, epidemiological analysis, e.g., in tracing contamination or the like. In particular, the analysis processes described above may be used to individually characterize, sequence and/or identify large numbers of individual cells within a population. This characterization may then be used to assemble an overall profile of the originating population, which can provide important prognostic and diagnostic information.

For example, shifts in human microbiomes, including, e.g., gut, buccal, epidermal microbiomes, etc., have been identified as being both diagnostic and prognostic of different conditions or general states of health. Using the single cell analysis methods and systems described herein, one can again, characterize, sequence and identify individual cells in an overall population, and identify shifts within that population that may be indicative of diagnostic ally relevant factors. By way of example, sequencing of bacterial 16S ribosomal RNA genes has been used as a highly accurate method for taxonomic classification of bacteria. Using the targeted amplification and sequencing processes described above can provide identification of individual cells within a population of cells. One may further quantify the numbers of different cells within a population to identify current states or shifts in states over time. See, e.g., Morgan et al, PLoS Comput. Biol., Ch. 12, December 2012, 8(12):e1002808, and Ram et al., Syst. Biol. Reprod. Med., June 2011, 57(3):162-170, each of which is incorporated herein by reference in its entirety for all purposes. Likewise, identification and diagnosis of infection or potential infection may

also benefit from the single cell analyses described herein, e.g., to identify microbial species present in large mixes of other cells or other biological material, cells and/or nucleic acids, including the environments described above, as well as any other diagnostically relevant environments, e.g., cerebrospinal fluid, blood, fecal or intestinal samples, or the like.

The foregoing analyses may also be particularly useful in the characterization of potential drug resistance of different cells, e.g., cancer cells, bacterial pathogens, etc., through the analysis of distribution and profiling of different resistance markers/mutations across cell populations in a given sample. Additionally, characterization of shifts in these markers/mutations across populations of cells over time can provide valuable insight into the progression, alteration, prevention, and treatment of a variety of diseases characterized by such drug resistance issues.

Although described in terms of cells, it will be appreciated that any of a variety of individual biological organisms, or components of organisms are encompassed within this description, including, for example, cells, viruses, organelles, cellular inclusions, vesicles, or the like. Additionally, where referring to cells, it will be appreciated that such reference includes any type of cell, including without limitation prokaryotic cells, eukaryotic cells, bacterial, fungal, plant, mammalian, or other animal cell types, mycoplasmas, normal tissue cells, tumor cells, or any other cell type, whether derived from single cell or multicellular organisms.

Similarly, analysis of different environmental samples to profile the microbial organisms, viruses, or other biological contaminants that are present within such samples, can provide important information about disease epidemiology, and potentially aid in forecasting disease outbreaks, epidemics an pandemics.

As described above, the methods, systems and compositions described herein may also be used for analysis and characterization of other aspects of individual cells or populations of cells. In one example process, a sample is provided that contains cells that are to be analyzed and characterized as to their cell surface proteins. Also provided is a library of antibodies, antibody fragments, or other molecules having a binding affinity to the cell surface proteins or antigens (or other cell features) for which the cell is to be characterized (also referred to herein as cell surface feature binding groups). For ease of discussion, these affinity groups are referred to herein as binding groups. The binding groups can include a reporter molecule that is indicative of the cell surface feature to which the binding group binds. In particular, a binding group type that is specific to one type of cell surface feature will comprise a first reporter molecule, while a binding group type that is specific to a different cell surface feature will have a different reporter molecule associated with it. In some aspects, these reporter molecules will comprise oligonucleotide sequences. Oligonucleotide based reporter molecules provide advantages of being able to generate significant diversity in terms of sequence, while also being readily attachable to most biomolecules, e.g., antibodies, etc., as well as being readily detected, e.g., using sequencing or array technologies. In the example process, the binding groups include oligonucleotides attached to them. Thus, a first binding group type, e.g., antibodies to a first type of cell surface feature, will have associated with it a reporter oligonucleotide that has a first nucleotide sequence. Different binding group types, e.g., antibodies having binding affinity for other, different cell surface features, will have associated therewith reporter oligonucleotides that comprise different nucleotide sequences, e.g., having a partially or completely different nucleotide sequence. In some cases, for each type of cell surface feature binding group, e.g., antibody or antibody fragment, the reporter oligonucleotide sequence may be known and readily identifiable as being associated with the known cell surface feature binding group. These oligonucleotides may be directly coupled to the binding group, or they may be attached to a bead, molecular lattice, e.g., a linear, globular, cross-slinked, or other polymer, or other framework that is attached or otherwise associated with the binding group, which allows attachment of multiple reporter oligonucleotides to a single binding group.

In the case of multiple reporter molecules coupled to a single binding group, such reporter molecules can comprise the same sequence, or a particular binding group will include a known set of reporter oligonucleotide sequences. As between different binding groups, e.g., specific for different cell surface features, the reporter molecules can be different and attributable to the particular binding group.

Attachment of the reporter groups to the binding groups may be achieved through any of a variety of direct or indirect, covalent or non-covalent associations or attachments. For example, in the case of oligonucleotide reporter groups associated with antibody based binding groups, such oligonucleotides may be covalently attached to a portion of an antibody or antibody fragment using chemical conjugation techniques (e.g., Lightning-Link® antibody labeling kits available from Innova Biosciences), as well as other non-covalent attachment mechanisms, e.g., using biotinylated antibodies and oligonucleotides (or beads that include one or more biotinylated linker, coupled to oligonucleotides) with an avidin or streptavidin linker. Antibody and oligonucleotide biotinylation techniques are available (See, e.g., Fang, et al., *Fluoride-Cleavable Biotinylation Phosphoramidite for 5'-end-Labeling and Affinity Purification of Synthetic Oligonucleotides*, Nucleic Acids Res. Jan. 15, 2003; 31(2):708-715, DNA 3' End Biotinylation Kit, available from Thermo Scientific, the full disclosures of which are incorporated herein by reference in their entirety for all purposes). Likewise, protein and peptide biotinylation techniques have been developed and are readily available (See, e.g., U.S. Pat. No. 6,265,552, the full disclosures of which are incorporated herein by reference in their entirety for all purposes).

The reporter oligonucleotides may be provided having any of a range of different lengths, depending upon the diversity of reporter molecules desired or a given analysis, the sequence detection scheme employed, and the like. In some cases, these reporter sequences can be greater than about 5 nucleotides in length, greater than about 10 nucleotides in length, greater than about 20, 30, 40, 50, 60, 70, 80, 90, 100, 120, 150 or even 200 nucleotides in length. In some cases, these reporter nucleotides may be less than about 250 nucleotides in length, less than about 200, 180, 150, 120 100, 90, 80, 70, 60, 50, 40, or even 30 nucleotides in length. In many cases, the reporter oligonucleotides may be selected to provide barcoded products that are already sized, and otherwise configured to be analyzed on a sequencing system. For example, these sequences may be provided at a length that ideally creates sequenceable products of a desired length for particular sequencing systems. Likewise, these reporter oligonucleotides may include additional sequence elements, in addition to the reporter sequence, such as sequencer attachment sequences, sequencing primer sequences, amplification primer sequences, or the complements to any of these.

In operation, a cell-containing sample is incubated with the binding molecules and their associated reporter oligonucleotides, for any of the cell surface features desired to be analyzed. Following incubation, the cells are washed to remove unbound binding groups. Following washing, the cells are partitioned into separate partitions, e.g., droplets, along with the barcode carrying beads described above, where each partition includes a limited number of cells, e.g., in some cases, a single cell. Upon releasing the barcodes from the beads, they will prime the amplification and barcoding of the reporter oligonucleotides. As noted above, the barcoded replicates of the reporter molecules may additionally include functional sequences, such as primer sequences, attachment sequences or the like.

The barcoded reporter oligonucleotides are then subjected to sequence analysis to identify which reporter oligonucleotides bound to the cells within the partitions. Further, by also sequencing the associated barcode sequence, one can identify that a given cell surface feature likely came from the same cell as other, different cell surface features, whose reporter sequences include the same barcode sequence, i.e., they were derived from the same partition.

Based upon the reporter molecules that emanate from an individual partition based upon the presence of the barcode sequence, one may then create a cell surface profile of individual cells from a population of cells. Profiles of individual cells or populations of cells may be compared to profiles from other cells, e.g., 'normal' cells, to identify variations in cell surface features, which may provide diagnostically relevant information. In particular, these profiles may be particularly useful in the diagnosis of a variety of disorders that are characterized by variations in cell surface receptors, such as cancer and other disorders.

Methods of the disclosure may be applicable to processing samples for the detection of changes in gene expression. A sample may comprise a cell, mRNA, or cDNA reverse transcribed from mRNA. The sample may be a pooled sample, comprising extracts from several different cells or tissues, or a sample comprising extracts from a single cell or tissue. methods of the invention may be used to fragment and barcode the polynucleotides of the cell for

Cells may be placed directly into a partition (e.g., a microwell) and lysed. After lysis, the sequencing. Polynucleotides may also be extracted from cells prior to introducing them into a partition used in a method of the invention. Reverse transcription of mRNA may be performed in a partition described herein, or outside of such a partition. Sequencing cDNA may provide an indication of the abundance of a particular transcript in a particular cell over time, or after exposure to a particular condition.

V. DEVICES AND SYSTEMS

Also provided herein are the microfluidic devices used for partitioning the cells as described above. Such microfluidic devices can comprise channel networks for carrying out the partitioning process like those set forth in FIGS. **1** and **2**. Examples of particularly useful microfluidic devices are described in U.S. Provisional Patent Application No. 61/977,804, filed Apr. 4, 2014, and incorporated herein by reference in its entirety for all purposes. Briefly, these microfluidic devices can comprise channel networks, such as those described herein, for partitioning cells into separate partitions, and co-partitioning such cells with oligonucleotide barcode library members, e.g., disposed on beads. These channel networks can be disposed within a solid body, e.g., a glass, semiconductor or polymer body structure in which the channels are defined, where those channels communicate at their termini with reservoirs for receiving the various input fluids, and for the ultimate deposition of the partitioned cells, etc., from the output of the channel networks. By way of example, and with reference to FIG. **2**, a reservoir fluidly coupled to channel **202** may be provided with an aqueous suspension of cells **214**, while a reservoir coupled to channel **204** may be provided with an aqueous suspension of beads **216** carrying the oligonucleotides. Channel segments **206** and **208** may be provided with a non-aqueous solution, e.g., an oil, into which the aqueous fluids are partitioned as droplets at the channel junction **212**. Finally, an outlet reservoir may be fluidly coupled to channel **210** into which the partitioned cells and beads can be delivered and from which they may be harvested. As will be appreciated, while described as reservoirs, it will be appreciated that the channel segments may be coupled to any of a variety of different fluid sources or receiving components, including tubing, manifolds, or fluidic components of other systems.

Also provided are systems that control flow of these fluids through the channel networks e.g., through applied pressure differentials, centrifugal force, electrokinetic pumping, capillary or gravity flow, or the like.

VI. KITS

Also provided herein are kits for analyzing individual cells or small populations of cells. The kits may include one, two, three, four, five or more, up to all of partitioning fluids, including both aqueous buffers and non-aqueous partitioning fluids or oils, nucleic acid barcode libraries that are releasably associated with beads, as described herein, microfluidic devices, reagents for disrupting cells amplifying nucleic acids, and providing additional functional sequences on fragments of cellular nucleic acids or replicates thereof, as well as instructions for using any of the foregoing in the methods described herein.

VII. COMPUTER CONTROL SYSTEMS

The present disclosure provides computer control systems that are programmed to implement methods of the disclosure. FIG. **17** shows a computer system **1701** that is programmed or otherwise configured to implement methods of the disclosure including nucleic acid sequencing methods, interpretation of nucleic acid sequencing data and analysis of cellular nucleic acids, such as RNA (e.g., mRNA), and characterization of cells from sequencing data. The computer system **1701** can be an electronic device of a user or a computer system that is remotely located with respect to the electronic device. The electronic device can be a mobile electronic device.

The computer system **1701** includes a central processing unit (CPU, also "processor" and "computer processor" herein) **1705**, which can be a single core or multi core processor, or a plurality of processors for parallel processing. The computer system **1701** also includes memory or memory location **1710** (e.g., random-access memory, read-only memory, flash memory), electronic storage unit **1715** (e.g., hard disk), communication interface **1720** (e.g., network adapter) for communicating with one or more other systems, and peripheral devices **1725**, such as cache, other memory, data storage and/or electronic display adapters. The memory **1710**, storage unit **1715**, interface **1720** and peripheral devices **1725** are in communication with the CPU **1705** through a communication bus (solid lines), such as a moth-

erboard. The storage unit **1715** can be a data storage unit (or data repository) for storing data. The computer system **1701** can be operatively coupled to a computer network ("network") **1730** with the aid of the communication interface **1720**. The network **1730** can be the Internet, an internet and/or extranet, or an intranet and/or extranet that is in communication with the Internet. The network **1730** in some cases is a telecommunication and/or data network. The network **1730** can include one or more computer servers, which can enable distributed computing, such as cloud computing. The network **1730**, in some cases with the aid of the computer system **1701**, can implement a peer-to-peer network, which may enable devices coupled to the computer system **1701** to behave as a client or a server.

The CPU **1705** can execute a sequence of machine-readable instructions, which can be embodied in a program or software. The instructions may be stored in a memory location, such as the memory **1710**. The instructions can be directed to the CPU **1705**, which can subsequently program or otherwise configure the CPU **1705** to implement methods of the present disclosure. Examples of operations performed by the CPU **1705** can include fetch, decode, execute, and writeback.

The CPU **1705** can be part of a circuit, such as an integrated circuit. One or more other components of the system **1701** can be included in the circuit. In some cases, the circuit is an application specific integrated circuit (ASIC).

The storage unit **1715** can store files, such as drivers, libraries and saved programs. The storage unit **1715** can store user data, e.g., user preferences and user programs. The computer system **1701** in some cases can include one or more additional data storage units that are external to the computer system **1701**, such as located on a remote server that is in communication with the computer system **1701** through an intranet or the Internet.

The computer system **1701** can communicate with one or more remote computer systems through the network **1730**. For instance, the computer system **1701** can communicate with a remote computer system of a user. Examples of remote computer systems include personal computers (e.g., portable PC), slate or tablet PC's (e.g., Apple® iPad, Samsung® Galaxy Tab), telephones, Smart phones (e.g., Apple® iPhone, Android-enabled device, Blackberry®), or personal digital assistants. The user can access the computer system **1701** via the network **1730**.

Methods as described herein can be implemented by way of machine (e.g., computer processor) executable code stored on an electronic storage location of the computer system **1701**, such as, for example, on the memory **1710** or electronic storage unit **1715**. The machine executable or machine readable code can be provided in the form of software. During use, the code can be executed by the processor **1705**. In some cases, the code can be retrieved from the storage unit **1715** and stored on the memory **1710** for ready access by the processor **1705**. In some situations, the electronic storage unit **1715** can be precluded, and machine-executable instructions are stored on memory **1710**.

The code can be pre-compiled and configured for use with a machine having a processer adapted to execute the code, or can be compiled during runtime. The code can be supplied in a programming language that can be selected to enable the code to execute in a pre-compiled or as-compiled fashion.

Aspects of the systems and methods provided herein, such as the computer system **1701**, can be embodied in programming. Various aspects of the technology may be thought of as "products" or "articles of manufacture" typically in the form of machine (or processor) executable code and/or associated data that is carried on or embodied in a type of machine readable medium. Machine-executable code can be stored on an electronic storage unit, such as memory (e.g., read-only memory, random-access memory, flash memory) or a hard disk. "Storage" type media can include any or all of the tangible memory of the computers, processors or the like, or associated modules thereof, such as various semiconductor memories, tape drives, disk drives and the like, which may provide non-transitory storage at any time for the software programming. All or portions of the software may at times be communicated through the Internet or various other telecommunication networks. Such communications, for example, may enable loading of the software from one computer or processor into another, for example, from a management server or host computer into the computer platform of an application server. Thus, another type of media that may bear the software elements includes optical, electrical and electromagnetic waves, such as used across physical interfaces between local devices, through wired and optical landline networks and over various air-links. The physical elements that carry such waves, such as wired or wireless links, optical links or the like, also may be considered as media bearing the software. As used herein, unless restricted to non-transitory, tangible "storage" media, terms such as computer or machine "readable medium" refer to any medium that participates in providing instructions to a processor for execution.

Hence, a machine readable medium, such as computer-executable code, may take many forms, including but not limited to, a tangible storage medium, a carrier wave medium or physical transmission medium. Non-volatile storage media include, for example, optical or magnetic disks, such as any of the storage devices in any computer(s) or the like, such as may be used to implement the databases, etc. shown in the drawings. Volatile storage media include dynamic memory, such as main memory of such a computer platform. Tangible transmission media include coaxial cables; copper wire and fiber optics, including the wires that comprise a bus within a computer system. Carrier-wave transmission media may take the form of electric or electromagnetic signals, or acoustic or light waves such as those generated during radio frequency (RF) and infrared (IR) data communications. Common forms of computer-readable media therefore include for example: a floppy disk, a flexible disk, hard disk, magnetic tape, any other magnetic medium, a CD-ROM, DVD or DVD-ROM, any other optical medium, punch cards paper tape, any other physical storage medium with patterns of holes, a RAM, a ROM, a PROM and EPROM, a FLASH-EPROM, any other memory chip or cartridge, a carrier wave transporting data or instructions, cables or links transporting such a carrier wave, or any other medium from which a computer may read programming code and/or data. Many of these forms of computer readable media may be involved in carrying one or more sequences of one or more instructions to a processor for execution.

The computer system **1701** can include or be in communication with an electronic display **1735** that comprises a user interface (UI) **1740** for providing, for example, results of nucleic acid sequencing, analysis of nucleic acid sequencing data, characterization of nucleic acid sequencing samples, cell characterizations, etc. Examples of UI's include, without limitation, a graphical user interface (GUI) and web-based user interface.

Methods and systems of the present disclosure can be implemented by way of one or more algorithms. An algo-

rithm can be implemented by way of software upon execution by the central processing unit **1705**. The algorithm can, for example, initiate nucleic acid sequencing, process nucleic acid sequencing data, interpret nucleic acid sequencing results, characterize nucleic acid samples, characterize cells, etc.

VIII. EXAMPLES

Example I Cellular RNA Analysis Using Emulsions

In an example, reverse transcription with template switching and cDNA amplification (via PCR) is performed in emulsion droplets with operations as shown in FIG. **9**A. The reaction mixture that is partitioned for reverse transcription and cDNA amplification (via PCR) includes 1,000 cells or 10,000 cells or 10 ng of RNA, beads bearing barcoded oligonucleotides/0.2% Tx-100/5× Kapa buffer, 2× Kapa HS HiFi Ready Mix, 4 μM switch oligo, and Smartscribe. Where cells are present, the mixture is partitioned such that a majority or all of the droplets comprise a single cell and single bead. The cells are lysed while the barcoded oligonucleotides are released from the bead, and the poly-T segment of the barcoded oligonucleotide hybridizes to the poly-A tail of mRNA that is released from the cell as in operation **950**. The poly-T segment is extended in a reverse transcription reaction as in operation **952** and the cDNA transcript is amplified as in operation **954**. The thermal cycling conditions are 42° C. for 130 minutes; 98° C. for 2 min; and 35 cycles of the following 98° C. for 15 sec, 60° C. for 20 sec, and 72° C. for 6 min. Following thermal cycling, the emulsion is broken and the transcripts are purified with Dynabeads and 0.6× SPRI as in operation **956**.

The yield from template switch reverse transcription and PCR in emulsions is shown for 1,000 cells in FIG. **13**A and 10,000 cells in FIG. **13**C and 10 ng of RNA in FIG. **13**B (Smartscribe line). The cDNA transcripts from RT and PCR performed in emulsions for 10 ng RNA is sheared and ligated to functional sequences, cleaned up with 0.8×SPRI, and is further amplified by PCR as in operation **958**. The amplification product is cleaned up with 0.8×SPRI. The yield from this processing is shown in FIG. **13**B (SSII line).

Example II Cellular RNA Analysis Using Emulsions

In another example, reverse transcription with template switching and cDNA amplification (via PCR) is performed in emulsion droplets with operations as shown in FIG. **9**A. The reaction mixture that is partitioned for reverse transcription and cDNA amplification (via PCR) includes Jurkat cells, beads bearing barcoded oligonucleotides/0.2% TritonX-100/5× Kapa buffer, 2× Kapa HS HiFi Ready Mix, 4 μM switch oligo, and Smartscribe. The mixture is partitioned such that a majority or all of the droplets comprise a single cell and single bead. The cells are lysed while the barcoded oligonucleotides are released from the bead, and the poly-T segment of the barcoded oligonucleotide hybridizes to the poly-A tail of mRNA that is released from the cell as in operation **950**. The poly-T segment is extended in a reverse transcription reaction as in operation **952** and the cDNA transcript is amplified as in operation **954**. The thermal cycling conditions are 42° C. for 130 minutes; 98° C. for 2 min; and 35 cycles of the following 98° C. for 15 sec, 60° C. for 20 sec, and 72° C. for 6 min. Following thermal cycling, the emulsion is broken and the transcripts are cleaned-up with Dynabeads and 0.6× SPRI as in operation **956**. The yield from reactions with various cell numbers (625 cells, 1,250 cells, 2,500 cells, 5,000 cells, and 10,000 cells) is shown in FIG. **14**A. These yields are confirmed with GADPH qPCR assay results shown in FIG. **14**B.

Example III RNA Analysis Using Emulsions

In another example, reverse transcription is performed in emulsion droplets and cDNA amplification is performed in bulk in a manner similar to that as shown in FIG. **9**C. The reaction mixture that is partitioned for reverse transcription includes beads bearing barcoded oligonucleotides, 10 ng Jurkat RNA (e.g., Jurkat mRNA), 5× First-Strand buffer, and Smartscribe. The barcoded oligonucleotides are released from the bead, and the poly-T segment of the barcoded oligonucleotide hybridizes to the poly-A tail of the RNA as in operation **961**. The poly-T segment is extended in a reverse transcription reaction as in operation **963**. The thermal cycling conditions for reverse transcription are one cycle at 42° C. for 2 hours and one cycle at 70° C. for 10 min. Following thermal cycling, the emulsion is broken and RNA and cDNA transcripts are denatured as in operation **962**. A second strand is then synthesized by primer extension with a primer having a biotin tag as in operation **964**. The reaction conditions for this primer extension include cDNA transcript as the first strand and biotinylated extension primer ranging in concentration from 0.5-3.0 μM. The thermal cycling conditions are one cycle at 98° C. for 3 min and one cycle of 98° C. for 15 sec, 60° C. for 20 sec, and 72° C. for 30 min. Following primer extension, the second strand is pulled down with Dynabeads MyOne Streptavidin C1 and T1, and cleaned-up with Agilent SureSelect XT buffers. The second strand is pre-amplified via PCR as in operation **965** with the following cycling conditions—one cycle at 98° C. for 3 min and one cycle of 98° C. for 15 sec, 60° C. for 20 sec, and 72° C. for 30 min. The yield for various concentrations of biotinylated primer (0.5 μM, 1.0 μM, 2.0 μM, and 3.0 μM) is shown in FIG. **15**.

Example IV RNA Analysis Using Emulsions

In another example, in vitro transcription by T7 polymerase is used to produce RNA transcripts as shown in FIG. **10**. The mixture that is partitioned for reverse transcription includes beads bearing barcoded oligonucleotides which also include a T7 RNA polymerase promoter sequence, 10 ng human RNA (e.g., human mRNA), 5× First-Strand buffer, and Smartscribe. The mixture is partitioned such that a majority or all of the droplets comprise a single bead. The barcoded oligonucleotides are released from the bead, and the poly-T segment of the barcoded oligonucleotide hybridizes to the poly-A tail of the RNA as in operation **1050**. The poly-T segment is extended in a reverse transcription reaction as in operation **1052**. The thermal cycling conditions are one cycle at 42° C. for 2 hours and one cycle at 70° C. for 10 min. Following thermal cycling, the emulsion is broken and the remaining operations are performed in bulk. A second strand is then synthesized by primer extension as in operation **1054**. The reaction conditions for this primer extension include cDNA transcript as template and extension primer. The thermal cycling conditions are one cycle at 98° C. for 3 min and one cycle of 98° C. for 15 sec, 60° C. for 20 sec, and 72° C. for 30 min. Following this primer extension, the second strand is purified with 0.6×SPRI. As in operation **1056**, in vitro transcription is then performed to produce RNA transcripts. In vitro transcription is performed

overnight, and the transcripts are purified with 0.6×SPRI. The RNA yields from in vitro transcription are shown in FIG. **16**.

While some embodiments of the present invention have been shown and described herein, it will be obvious to those skilled in the art that such embodiments are provided by way of example only. It is not intended that the invention be limited by the specific examples provided within the specification. While the invention has been described with reference to the aforementioned specification, the descriptions and illustrations of the embodiments herein are not meant to be construed in a limiting sense. Numerous variations, changes, and substitutions will now occur to those skilled in the art without departing from the invention. Furthermore, it shall be understood that all aspects of the invention are not limited to the specific depictions, configurations or relative proportions set forth herein which depend upon a variety of conditions and variables. It should be understood that various alternatives to the embodiments of the invention described herein may be employed in practicing the invention. It is therefore contemplated that the invention shall also cover any such alternatives, modifications, variations or equivalents. It is intended that the following claims define the scope of the invention and that methods and structures within the scope of these claims and their equivalents be covered thereby.

What is claimed is:

1. A method of single cell gene expression analysis, comprising:

(a) providing a plurality of beads, wherein a bead of said plurality of beads comprises a plurality of nucleic acid barcode molecules each comprising:

(i) a common barcode sequence configured to identify a cellular origin of mRNA molecules; and

(ii) an identification barcode sequence configured to quantify mRNA molecules;

(b) partitioning a plurality of cells and said plurality of beads in a system comprising a plurality of partitions, wherein said system comprises 1,000 occupied partitions each comprising a single cell of said plurality of cells and a single bead of said plurality of beads, wherein different occupied partitions have different common barcode sequences;

(c) releasing mRNA molecules from single cells of said 1,000 occupied partitions;

(d) using said plurality of nucleic acid barcode molecules to generate a plurality of barcoded nucleic acid molecules from the mRNA molecules released in (c), wherein a barcoded nucleic acid molecule of said plurality of barcoded nucleic acid molecules comprises: (i) a sequence of an mRNA molecule of said released mRNA molecules, or a reverse complement of said sequence of said mRNA molecule; and (ii) a sequence of a nucleic acid barcode molecule of said plurality of nucleic acid barcode molecules, including said common barcode sequence and said identification barcode sequence, or a reverse complement of said sequence of said nucleic acid barcode molecule;

(e) determining sequences of said plurality of barcoded nucleic acid molecules;

(f) using common barcode sequences from said sequences determined in (e) to identify cellular origins of said plurality of barcoded nucleic acid molecules, thereby identifying cellular origins of said mRNA molecules from which said plurality of barcoded nucleic acid molecules were generated; and

(g) using identification barcode sequences from said sequences determined in (e) to identify molecular origins of said plurality of barcoded nucleic acid molecules, thereby quantifying said mRNA molecules from which said plurality of barcoded nucleic acid molecules were generated.

2. The method of claim **1**, wherein said system comprises 5,000 occupied partitions.

3. The method of claim **1**, wherein said system comprises 10,000 occupied partitions.

4. The method of claim **1**, wherein no more than 5% of said 1,000 occupied partitions have more than one cell.

5. The method of claim **1**, wherein no more than 1% of said 1,000 occupied partitions have more than one cell.

6. The method of claim **1**, wherein no more than 5% of said 1,000 occupied partitions have more than one bead.

7. The method of claim **1**, wherein no more than 1% of said 1,000 occupied partitions have more than one bead.

8. The method of claim **1**, wherein at least 10% of said plurality of partitions are occupied partitions.

9. The method of claim **1**, wherein at least 50% of said plurality of partitions are occupied partitions.

10. The method of claim **1**, wherein said releasing in (c) comprises lysing said single cells of said 1,000 occupied partitions.

11. The method of claim **1**, wherein said nucleic acid barcode molecule of said plurality of nucleic acid barcode molecules comprises a poly-T sequence for capturing mRNA molecules, and (d) comprises hybridizing said poly-T sequence to a poly-A tail of said mRNA molecule of said released mRNA molecules.

12. The method of claim **11**, wherein (d) comprises performing a reverse transcription reaction using said nucleic acid barcode molecule as a primer, thereby generating a complementary deoxyribonucleic acid (cDNA) molecule that incorporates at least a portion of said nucleic acid barcode molecule.

13. The method of claim **1**, wherein (d) comprises ligating said nucleic acid barcode molecule to said mRNA molecule.

14. The method of claim **1**, further comprising pooling barcoded nucleic acid molecules derived from different cells prior to (e).

15. The method of claim **14**, wherein (d) further comprises performing two or more polymerase chain reactions (PCR) subsequent to said pooling.

16. The method of claim **1**, wherein (e) comprises determining said sequences of said plurality of barcoded nucleic acid molecules using a sequencer.

17. The method of claim **1**, wherein (e) comprises determining said sequences of said plurality of barcoded nucleic acid molecules using an array.

18. The method of claim **1**, wherein said plurality of nucleic acid barcode molecules comprises 1,000,000 different common barcode sequences.

19. The method of claim **1**, wherein said plurality of nucleic acid barcode molecules comprises 10,000,000 different common barcode sequences.

20. The method of claim **1**, wherein said plurality of nucleic acid barcode molecules comprises common barcode sequences that are modular sequences.

21. The method of claim **1**, wherein said plurality of nucleic acid barcode molecules comprises 100,000 different identification barcode sequences.

22. The method of claim **1**, wherein said plurality of nucleic acid barcode molecules comprises 1,000,000 different identification barcode sequences.

23. The method of claim **1**, wherein said plurality of nucleic acid barcode molecules comprises identification barcode sequences that are random sequences.

24. The method of claim **1**, wherein said system is a microwell array and said plurality of partitions is a plurality of wells.

25. The method of claim **24**, wherein said microwell array comprises 100,000 wells.

26. The method of claim **24**, wherein a well of said microwell array has a volume of less than about 5 nanoliters (nL).

27. The method of claim **1**, wherein said system is a droplet microfluidic system and said plurality of partitions is a plurality of droplets.

28. The method of claim **1**, wherein said plurality of beads is a plurality of gel beads.

29. The method of claim **1**, wherein said plurality of beads is a plurality of magnetic beads.

30. The method of claim **1**, wherein an occupied partition of said 1,000 occupied partitions comprises an antibody.

31. The method of claim **24**, wherein said microwell array further comprises an inlet port in fluid communication with said plurality of wells.

32. The method of claim **31**, wherein said microwell array further comprises an outlet port in fluid communication with said plurality of wells.

33. The method of claim **32**, wherein said partitioning comprises flowing a solution from said plurality of wells via said outlet port.

34. The method of claim **31**, wherein said partitioning comprises flowing said plurality of cells to said plurality of wells via said inlet port.

35. The method of claim **24**, wherein said plurality of wells are hexagonal in cross- sectional dimension.

36. The method of claim **1**, wherein said plurality of nucleic acid barcode molecules are releasably attached to said plurality of beads.

37. The method of claim **1**, wherein said plurality of nucleic acid barcode molecules are attached to said plurality of beads via one or more linkers.

38. The method of claim **37**, wherein said one or more linkers comprise a disulfide moiety.

39. The method of claim **1**, wherein said plurality of nucleic acid barcode molecules each comprises a primer binding sequence.

40. The method of claim **1**, wherein (d) is performed in said plurality of partitions.

41. The method of claim **1**, wherein (d) is performed outside said plurality of partitions.

42. The method of claim **41**, wherein, prior to (d), contents of said plurality of partitions are pooled.

43. The method of claim **1**, wherein said mRNA molecule of said released mRNA molecules is hybridized to said nucleic acid barcode molecule of said plurality of nucleic acid barcode molecules, thereby attaching said mRNA molecule to said bead, and said bead is removed from said partition prior to (d).

44. The method of claim **43**, wherein said nucleic acid barcode molecule comprises a poly-T sequence, and wherein a poly-A sequence of said mRNA molecule is hybridized to said poly-T sequence, thereby attaching said mRNA molecule to said bead.

45. The method of claim **41**, wherein a mRNA molecule of said mRNA molecules is released from a single cell of said single cells in a partition of said 1,000 occupied partitions during said partitioning in (b).

46. The method of claim **41**, wherein a mRNA molecule of said mRNA molecules is released from a single cell of said single cells in a partition of said 1,000 occupied partitions subsequent to said partitioning in (b).

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